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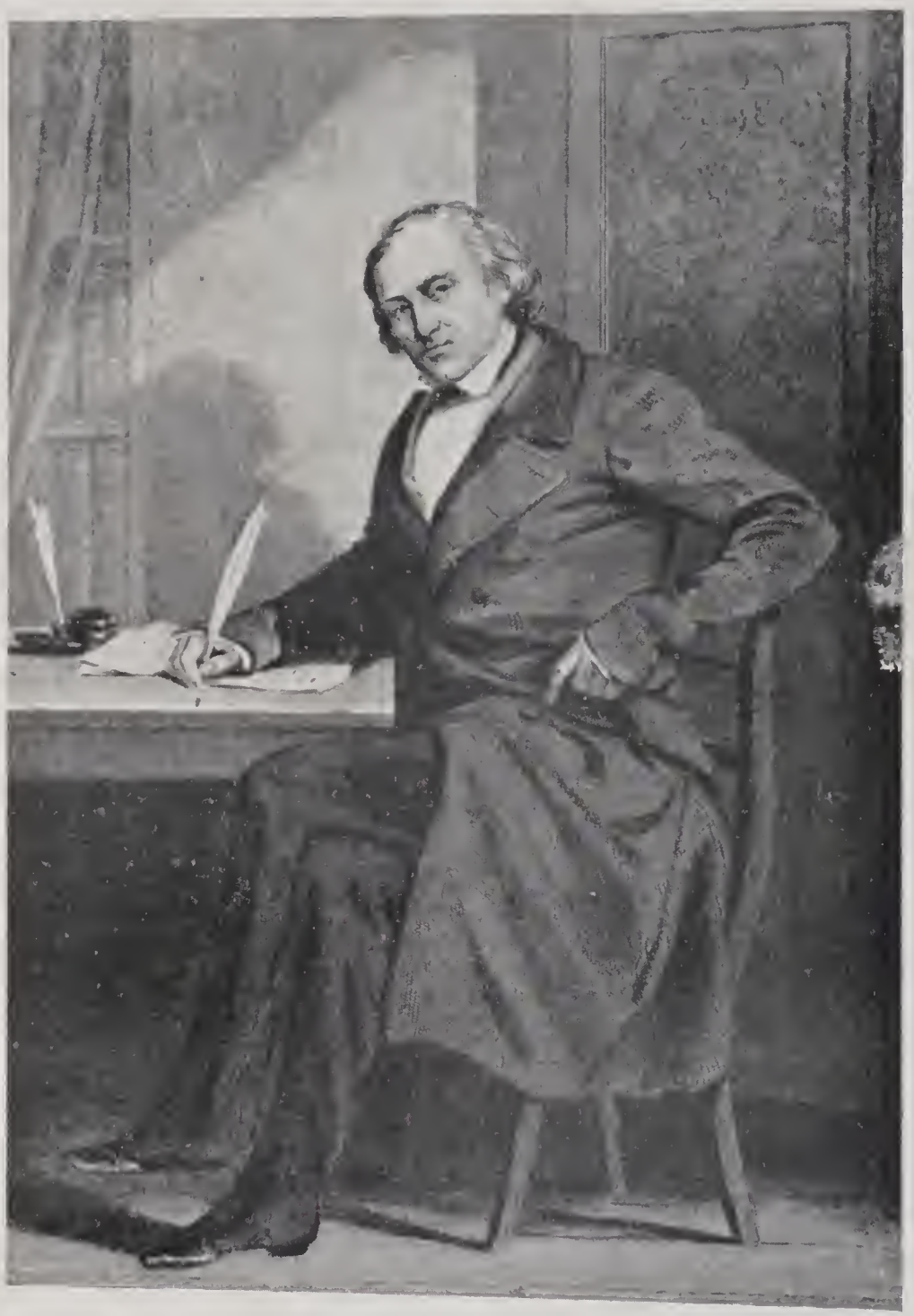

FRANCOIS-VINCENT RASPAIL
1794–1878
The founder of histochemistry

HISTOCHEMISTRY

Theoretical and Applied

HISTOCHEMISTRY

Theoretical and Applied

by

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SECOND EDITION

With 10 coloured plates

and

245 black and white illustrations

LONDON
J. & A. CHURCHILL, LTD.
104 GLOUCESTER PLACE, W.1
1960

To all those, histochemists and others, without whose help and without whose work this book could not have been written.

NAM PROPRIUM EST NIHIL.

Hor. Epist. i, II, 23 (apud Fcam).

First Edition 1953
Reprinted 1954
Translated into Polish
Translated into Spanish
Translated into Russian
Second Edition 1960

4229
GTX N60

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PREFACE

Since the preparation of the first edition of this book, and its publication in 1953, Histochemistry has notably expanded the range of its techniques. That is my excuse for the increased size of this volume. The application of Histochemistry to the descriptive sciences of biology, cytology, histology, pathology and zoology has increased in similar fashion during the past 6 years so that it is now difficult for a single individual to keep pace with its widespread literature.

The book, once again, represents an endeavour to compass the whole of histochemistry as applied to tissue sections and again it has been my endeavour to practise all the available methods in the various fields and to reject or modify those which proved defective. This has been done, but where any method is included which has not been tried in my laboratory, the fact is recorded in the text. For each method I have provided full theoretical details and sufficient critical comments to make clear its essential purpose and range of usefulness. Details of the modern applications of histochemistry to various disciplines have been described throughout the text but special chapters are devoted to the Scleroproteins and to Amyloid.

The section of the book devoted to enzyme histochemistry has expanded in keeping with the rapid rate of development in this field and the methods of Immunohistology now command a chapter of their own.

For the methods themselves the system of providing technical appendices to each chapter has met with general approval and is therefore continued. Although the appendices together form a complete and autonomous unit it is necessary to warn its users that occasional reference to the text may help them to avoid unnecessary labour in the use of unsuitable methods.

As in the preface to the first edition I make no apologies for the amounts of chemistry and biochemistry which appear in the text. On these sciences Histochemistry is based and only with their assistance can it continue to advance.

The extension of Histochemistry is essential in all those fields to which it is applicable. It can certainly transform the descriptive sciences into dynamic and functional ones, as has been increasingly demonstrated in the past few years. There is a growing awareness of the value of Histochemistry in extending the limits of the sciences to which its methods are applied and little doubt that this awareness

will bring in its train an ever increasing volume of applied histochemistry. If this second edition of my book provides the stimulus and the means for further extensions, I shall be amply rewarded.

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Hertfordshire.

ACKNOWLEDGEMENTS

As with the first edition I should like to begin by acknowledging my great indebtedness to Professor J. H. Dible, at whose suggestion I originally undertook this work, for his continued support of my histochemical endeavours. I would like to thank all my colleagues in the Department of Pathology at the Postgraduate Medical School for their help and tolerance, especially Professors E. J. King and C. V. Harrison and Drs. I. Doniach and W. Klyne.

Throughout the writing of this edition I have been notably assisted by those who have shared the work of my laboratory. In addition to my Technician Mr. B. C. S. Hollands, and my Research Assistant Mrs. Sheila Teller, I would like to acknowledge my corporate debt to Drs. de Almeida, Barter, Bontke, Burton, Defendi, Dubowitz, Grogg, Guerrero, Hess, Macpherson, Mayersbach, Niemi, Niwelinski, Oettle, Pepler, Portugalov, Przelecka, Rudolph, Scarpelli, Schor, Tremblay and Zimmermann. I also acknowledge the special assistance received from Dr. M. Niemi in the case of chapter XXVIII, from Dr. A. G. Oettle in the case of chapter XXVI and from Dr. D. G. Scarpelli in the preparation of chapters XIX and XX.

The whole work has been made easier by the full support given to me by many friends in the field of histochemistry. From Dr. A. M. Seligman I have received many gifts of reagents and enzyme substrates. I have been similarly helped by Professor Hans Beyer, Dr. M. F. Burstone, Dr. G. Glenner, Dr. E. Kambli and Dr. A. W. Nineham, and also by Imperial Chemical Industries Ltd. (Dyestuffs Division) and CIBA Ltd., Basel.

I am particularly indebted to Mrs. Helen Tremblay for her invaluable assistance in reading the proofs and making the author index, and to Miss D. F. Atkins and the Library Staff at the Postgraduate Medical School for their help in obtaining and checking references.

I would like to thank Mrs. B. Lamb, of Evershed and Vignoles, for her contribution to the successful polarography of a series of tetrazolium salts, and Dr. R. C. Valentine for help with the electron microscopy of formazans.

The photomicrographs are the work of many hands. Those surviving from the first edition were made by Mr. E. V. Willmott and the newer ones are due to Mr. W. Brackenbury, Mr. C. A. P. Graham, Mr. T. Hinton, Mrs. Gladys McQueen and Mrs. Rayner Wolff. The original drawings and equations were made by Miss S. Tomkins and the newer ones by Mr. F. G. Saunders.

The chapter on cold microtomes has been facilitated by a grant from the Central Council for the Care of Cripples for the development of the cryostat and for technical assistance. I should like to thank

Mr. C. Lorden and Mr. L. V. Farr for their help in the construction of the prototype. My work in the dehydrogenase field has been greatly helped by grants for materials and assistance from the Medical Research Council.

I also wish to thank the following authors, editors, and others for permission to use the undermentioned illustrations, some of which have appeared elsewhere in print : Fig. 1, Dr. N. Shimizu ; Figs. 2 and 3, Dr. Wayne Thornberg and the Editor, *Journal of Histochemistry and Cytochemistry* ; Figs. 11 and 15, Mr. L. J. Wright ; Figs. 12, 13 and 14, Professor Olavi Eränkö ; Fig. 17, Dr. O. E. Pratt and the Editor, *Acta Histochemica* ; Fig. 20, Dr. W. A. Jensen ; Figs. 47 and 48, The Editor, *British Journal of Experimental Pathology* ; Fig. 49, Dr. R. G. White and the Editor, *British Journal of Experimental Pathology* ; Fig. 85, Mr. K. C. Moreman, the British Medical Association and the Editor, *Journal of Clinical Pathology* ; Figs. 86 and 87, Dr. N. O. Berg and the Editor, *Acta Pathologica Scandinavica* ; Fig. 102, Dr. M. M. Nachlas ; Fig. 104, Professor M. Chèvremont ; Figs. 106 and 107, Dr. P. Loustalot ; Fig. 112, The Editor *Biochemical Journal* ; Fig. 169, Dr. M. H. Burstone ; Figs. 202, 203 and 237, Professor Olavi Eränkö ; Figs. 234 and 235, Dr. S. R. Pelc ; Fig. 238, Dr. O. Hallén ; Fig. 243, Dr. R. J. Barrnett ; Fig. 245, Dr. R. H. Herz and Messrs Kodak Ltd. ; Plate IIB, Dr. C. W. M. Adams and the Editor, *Journal of Pathology and Bacteriology* ; Plate IIIA, Dr. F. Seitelberger and Professor J. N. Cumings ; Plates IVC and VD, Dr. W. Goessner and the Editors, *Histochemie* ; Plates VIB and VIC, Dr. W. H. Fishman and the Editor, *Journal of Histochemistry and Cytochemistry* ; Plate VIIIA, Dr. M. H. Burstone and the Editor, *Journal of Histochemistry and Cytochemistry* ; Plate VIIIB, Drs. J. R. Penney and Bridget M. Balfour and the Editor, *Journal of Pathology and Bacteriology* ; Plate IX, the Editor, *Acta Histochemica* ; Plates XA and B, Dr. J. D. Hicks and the Editor, *Journal of Pathology and Bacteriology* ; Plates XC, D and E, Professor Sir Howard Florey and Dr. Margaret A. Jennings. For the reproduction of the portrait of Raspail, which forms the Frontispiece, I am indebted to the kindness of Miss A. M. Vidal-Hall, of the Scientific Office of the French Embassy.

Although I have chosen not to alter the dedication of this volume it is nevertheless solely on account of the tremendous volume of support given to me by my wife, Dr. Elizabeth Pearse, during the $4\frac{1}{2}$ years spent in preparing this edition, that I have been enabled to carry it through to the final stage of publication. She has undertaken the whole of the secretarial work, together with the proof reading and the preparation of the subject index. She has shared with Mrs. Helen Tremblay the onerous task of checking the references and preparing the author index. My gratitude to her cannot be expressed in the printed word.

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CHAPTER I

THE HISTORY OF HISTOCHEMISTRY

THERE is little difference of opinion about the antiquity of histochemistry, "a science as old as histology itself" according to Lison. The schism occurs when the continuity of the old with the newer histochemistry is considered. On the one hand we have the view, put forward by Lison in the first edition of his book, that the modern science differs profoundly from that of 100 years ago. "It is so different," he said, "that one can say it is entirely recent—created *de novo*." On the other hand there is the view, to which I myself subscribe, that the progress of histochemistry has been uninterrupted, and that its aims and principles to-day are essentially the same as they were in the past. This is not to say that alterations and improvements of the widest variety have not taken place. Histochemistry can seldom proceed faster, however, than the sister sciences of chemistry and biochemistry allow. On these two it depends for inspiration and guidance.

The historical survey given here appeared in shorter form in a review of modern methods in histochemistry (Pearse, 1951). It is designed to give a true impression of the continuity of what is at the same time the youngest and one of the oldest of the biological sciences. Below, as an introduction, appears a brief tabulated history. This is by no means complete, but it forms a skeleton on which to hang the otherwise unconnected individual references which follow.

1800–29 Isolated reports of the investigation of chemical as opposed to morphological structure in tissue preparations. Histochemistry unknown as a separate science.

1830–55 These years saw the beginnings of histochemistry as a science. In its origins it was primarily botanical ; for some decades the whole practice of histochemistry in its true sense was in the hands of the botanists. Various works on the subject appeared : Raspail's *Essai de Chimie Microscopique Appliquée à la Physiologie*, 1830 ; his *Nouveau Système de Chimie Organique*, 1833 ; Lehmann's *Lehrbuch der physiologischen Chemie*, 1842 ; Raspail's *New System of Organic Chemistry*, 1834 (translation).

1856–71 Histochemistry, in the case of animal as opposed to plant tissues, was mainly biological chemistry, and most of its methods involved tissue destruction. More textbooks on

this aspect of histochemistry began to appear, some of them being translations of earlier works : *Physiological Chemistry* of Lehmann, 1851 (translation) ; *Traité de Chimie Anatomique* by Robin and Verdeil, 1853 ; *Chemie der Gewebe des gesammten Thierreichs* of Schlossberger, 1856 ; *Handbuch der Experimental Physiologie der Pflanzen* of Sachs, 1865 ; and the *Handbuch der Histologie und Histochemie des Menschen* of Frey, 1867.

1872–98 At this stage histochemistry became divided, part left histology and became attached to physiology and part remained as biological chemistry. Articles and books published during this period included *Ein Beitrag zur Histochemie*, by Miescher, 1874 ; *Histology and Histochemistry of Man*, by Frey, 1874 (translation) ; *Traité de Chimie Physiologique* of Lehmann, 1883 (translation) ; Bunge's *Lehrbuch der physiologischen und pathologischen Chemie*, 1887 ; and Sachs' *Lectures on the Physiology of Plants*, 1887.

1899–1929 During this period the use of aniline dyes in histology, first described by Bencke in 1862, became widespread. The first quarter of the twentieth century saw the rapid expansion of descriptive histopathology. Histologists became more interested in new dyes and staining techniques and showed less interest in the chemistry of tissue structures. Although diagnostic significance was attached to many of the new colour reactions, no attempt was made to put them on a physical or chemical basis. Morphological studies overwhelmed histochemistry and Mann (1902) was rash enough to say that the study of mammalian micro-anatomy was " almost complete " by 1900. Three contributions of this period described microchemistry and microphysiology ; they were Mann's *Physiological Histology*, 1902, Ehrlich's *Encyclopädie der Mikroskopischen Technik*, 1903, and Macallum's *Methoden und Ergebnisse der Mikrochemie*, 1908, all classical works. Prenant (1910) reviewed the general state of histochemistry in a valuable paper, and Molisch's *Mikrochemie der Pflanzen*, 1913, contained much of histochemical and cytochemical interest. Other works published towards the close of this period, which show that histochemistry still flourished, were the *Review of Recent Developments in Histochemistry*, by Parat, 1927 ; *Animale Histochemie*, by Patzelt, 1928 ; *Prakticum der Histochemie*, by Klein, 1929 ; and *Histochemische Methoden*, by Hertwig, 1929.

1930–44 This stage saw the rebirth of histochemistry and its partial return to the domain of histology. The most important work of the period was Lison's *Histochemie Animale* (1936) in which the author proclaimed the new science of histochemistry without tissue destruction. It is impossible to over-estimate the effect of this book upon the progress and practice of histochemistry. It remained, until superseded in 1953 by a second edition of broader scope, the acknowledged bible of histochemists in all fields of the basic sciences. Other excellent, though less important, publications were the *Handbook of Chemical Microscopy*, by Chamot and Mason, 1930 ; *Histochemische Methoden* of Romeis, 1932 ; *Die Mikroveraschung als histochemische Hilfsmethode*, by Policard and Okkels, 1932 ; and Linderstrøm-Lang's *Problems in Histochemistry*, 1936.

1945–56 The first published work of this period was Glick's *Techniques of Histo- and Cytochemistry*, 1949. This dealt somewhat briefly with the theory and practice of histochemistry, as it concerns the histologist. In it was reviewed the entire compass of histochemistry, physiological, physical and histological, and much of the technical information was beyond the scope of any but specialists in small individual fields. After a brief interval there appeared four works largely or wholly devoted to histochemistry. These, in order of appearance, were Gomori's *Microsopic Histochemistry*, 1952 ; Danielli's *Cytochemistry*, 1953 ; the first edition of this work, 1953 ; and Lison's second edition *Histochimie et Cytochimie Animales*, 1953. Bourne's *Functional Histology*, 1953, though not intended as a histochemical treatise, was based largely on the application of histochemical techniques, and Lillie's second edition with its new title *Histopathologic Technic and Practical Histochemistry*, 1954, represents an important addition to histochemical literature though only partly devoted to this subject. A stimulating and critical review of modern histochemistry was given by Vialli in his *Introduzione alla Ricerca in Istochimica*, 1955, in which the merits and demerits of most aspects of the science were discussed. Evidence of the expanding outlook of modern histochemistry was offered by Mellor's *Analytical Cytology*, 1955, a work of many authors concerned with all types of microscopy and their application, and by Eränkö's *Quantitative Methods in Histology and Microscopic Histochemistry*, 1955. These two works stress particularly the quantitative side of histochemistry, whose future development is closely

bound up with progress in this direction. The rapidly developing science of histochemistry, which until 1950 had no journals or periodicals devoted to it, now has no less than seven. If one counts those publications in which histochemical or cytochemical papers form a substantial part or even a majority of the contributions the number is even larger. The main histochemical and cytochemical journals are, with dates of first publication, *Experimental Cell Research* (1950), *Journal of Histochemistry and Cytochemistry* (1953), *Acta Histochemica* (1954), *Journal of Biophysical and Biochemical Cytology* (1955), *Rivista di Istochimica* (1955), *Annales d'Histochimie* (1956), *Histochemie* (1958).

In a series of papers entitled *Contributions to the History of Microchemistry* Harms (1931–32) gave a detailed account of the histochemical works of the French pharmacist, botanist and microscopist, Franzçois-Vincent Raspail (1794–1878), and he concluded, with the support of other competent observers, that Raspail should be regarded as the founder of the science of Microchemistry. These views were carried a stage further by Baker (1943, 1945) in a monograph of the Quekett Microscopical Club and in the first edition of his book on cytological technique. He too considered that Raspail was the real founder of Histochemistry. His claims to that title I regard as unassailable and his portrait therefore forms the frontispiece of this book. It shows Raspail as a young man, at the time when his greatest work was being done, and before he deserted the realms of botany and histochemistry for the field of politics. The first clear appreciations of the science of microscopic tissue chemistry undoubtedly came from Raspail (1825a and b, 1829). This author, after formulating his four resolutions (quoted in full by Harms, 1931, and partly also by Baker, 1943), settled down to a study of the processes of fertilization in flowers and fruits of the *Graminaceæ*. The most important reaction which he used for this purpose was the iodine reaction for starch, first described by Colin and de Claubry in 1814, and by Stromeyer (1815), and employed in a microscopic study of starch grains by Caventou in 1826. It is not certain whether priority for the histochemical use of iodine solutions should go to Raspail or to Caventon ; the point is not of great importance since the latter did not pursue his histochemical studies any further. Raspail, on the other hand, discovered and applied many other histochemical reactions which are still of importance to-day. In 1829 he used the xanthoproteic reaction for protein and the hydrochloric acid (furfural) test for carbohydrate which became more widely known as the reaction of Liebermann (1887) ; the latter is not now employed in animal histochemistry. In applying sulphuric acid

to his plant tissues to demonstrate the presence of protein he was, in fact, using the aldehyde method for tryptophan which, modified by many workers since his time, is still applied in histochemistry as the Voisenet-Fürth reaction. According to Reichl (1889) this type of benzylidene condensation reaction was used by Mikosch to demonstrate protein in plant tissues with a mixture of benzaldehyde, sulphuric acid and ferric sulphate.

Raspail is now usually credited with the discovery of micro-incineration (in 1829 according to Baker). He was also the first person to study the pH of protoplasm, using an indicator dye, turnsole, obtained from a species of sun spurge found in the Mediterranean region. This dye, normally blue, turned pink in acid solution.

Almost contemporary with Raspail's discoveries were those of several other botanists who published accounts of true histochemical reactions. Amongst these the work of Mohl (1931) on the iodine reaction, and of Schleiden (1838) on the iodine-sulphuric acid reaction may be mentioned. Apart from botanical work, however, progress in histochemistry was very slow and little work which can be described as histochemical, even in the broadest sense, was recorded until after 1860. Among the oldest published techniques are those for demonstrating iron, and some of these had their origin in the work of Vogel (1845, 1847), who detected iron in the tissues by its conversion to black ferrous sulphide with yellow sulphide of ammonia. In 1867 Perls introduced his Prussian-blue method for demonstrating iron which remains the method of choice up to the present day. Perls was followed in 1868 by Quinke, who used Vogel's sulphide method, and his technique also survives to the present day in practically unmodified form. In 1844 Millon described his reaction for proteins which Hoffmann, in 1853, showed was actually a test for tyrosine. This reaction was not used in histochemistry until 1888 (by Leitgeb), although Payen (1843) had already demonstrated nitrogenous substances in vegetable tissues with mercury proto-nitrate. The first recorded localization of starch in the chloroplast was by Sachs (1887) in his monograph on Plant Physiology.

In 1850, though the result was not published until 1859, Claude Bernard performed his celebrated experiment by the injection into dogs of iron lactate and potassium ferrocyanide, locating the resulting Prussian blue, which developed in the presence of acid, not only in the gastric glands but on the surface of the gastric mucosa. Such *in vivo* techniques really belong to the domain of physiology but this particular example may justly be claimed for histochemistry. In 1850, also, Schulze first demonstrated his chlor-zinc-iodine method for cellulose. A description of this method, which is still in use to-day, was given by Fürnrohr (1850).

The use of enzymes for tissue digestion was first reported in 1861,

by Beale, who used gastric juice in order to remove unwanted tissues from the nerve fibres which he was studying. As such, the technique was really micro-anatomical, but it developed by the end of the century into a well-recognized technique described as enzymal analysis (Kossel and Mathews, 1898).

Demonstration of the presence of enzymes in tissue began with the work of Klebs (1868) and Struve (1872), both of whom showed that tincture of guaiac gave a blue colour with pus, thus first recording the presence of peroxidases now well known to occur in the granules of the leucocytes. Brandenburg (1900) first demonstrated the peroxidase reaction in the latter site. Cytochrome oxidase was first demonstrated by Ehrlich (1885), though not, of course, under that name. He performed the " Nadi " reaction (p. 511) *in vivo* by injecting α-naphthol and *p*-phenylenediamine into animals, observing the formation of indophenol blue in situations where " Nadi oxidase " was present.

Besides some of the methods mentioned above, during the period 1856–98 a number of other histochemical methods appeared. Heidenhain, in 1868, showed that ergastoplasm, the deeply basophil substance at the base of secreting gland cells, contained a material which could be precipitated with acetic acid. This is now recognized as ribonucleic acid. In 1870 he described the development of a brown colour in certain cells of the adrenal medulla when these were treated with chromic acid and this phenomenon is now called the chromaffin reaction. According to Lison, its discovery should be attributed to Henle in 1865. The use of enzymes for digestion is recorded during this period by Miescher (1871), who employed pepsin to free nuclei from cytoplasmic material, and by Stirling (1875) who isolated elastic fibres by means of digestion with gastric juice. These two were hardly histochemical techniques in the modern sense, but they serve to illustrate the destructive nature of much of the research into the chemistry of the tissues which was taking place at that time. It was this destructive element which caused Lison to separate the older histochemistry from his new non-destructive science.

In 1873 Miescher isolated nuclear chromatin by making use of its selective affinity for methyl green, and Ehrlich, in 1878–79, observed the effects of heat coagulation in increasing the affinity of hæmoglobin for nitro dyes. This last work finds a modern echo in inquiry into the effects of denaturation on the combination of dyes or histochemical reagents with specific groups in the tissue proteins. During this same period (1856–98) the aniline dyes came into general histological use, following their introduction into the field by Bencke (1862). This caused a revolution in the practice of histology and provided a check to the progress of histochemistry. The various dyes were largely used without any attempt to correlate their performance with the

chemical nature of the tissue components that were stained, although every endeavour was made to record the correlation of colour with structure in the histological sense. Notwithstanding this criticism, a considerable amount of work was done by a few authors in order to find out how the various stains attached themselves to the tissues. The physical theory of staining was upheld by Witt (1890–91) and particularly strongly by Fischer (1899), who explained all staining on the basis of absorption. Miescher and Ehrlich, and also Knecht (1888), believed that staining was a chemical process. Mann's (1902) comment on all this is interesting. The object of staining, he says, is first to determine morphological facts and second, " to recognize microchemically the existence and distribution of substances which we have been made aware of by macrochemical means." " It is not sufficient," he goes on, " to content ourselves with using acid and basic dyes and speculating on the basic or acid nature of the tissues, or to apply colour radicals with oxidizing or reducing properties ; but we must endeavour to find staining reactions which will indicate not only the presence of certain elements such as iron or phosphorus, but the presence of organic complexes such as the carbohydrate group, the nucleins, protamines, and others." These remarks show that Mann, at least, was aware of the problem, but few practical attempts to meet it were made by histologists in general.

Some of the reactions involving the use of aniline dyes were in fact histochemical, although their significance was often unappreciated or wrongly appreciated. In most cases the significance is still not fully understood. A reaction in this last category is the metachromatic staining of amyloid with methyl violet, first described by Cornil (1875). Almost simultaneously Heschl (1875) and Jürgens (1875) were working on general problems of metachromasia. Ehrlich's reaction for mast-cells, using a saturated alcoholic solution of dahlia containing 8 per cent. acetic acid, is essentially similar to modern methods using the thiazine dyes and may well be considered histochemical. Other reactions whose mechanism was poorly understood were the myelin methods of Weigert (1884) and Marchi (1892) and the anilin-violet method of Gram (1884). The chemical theory of staining was strongly supported by Mathews (1898) in his experimental work with albumins and albumoses and important researches into the nucleohistones were conducted by Saint-Hilaire (1898), who noticed, in his attempts to evolve a method for uric acid in the tissues, that nuclei were occasionally stained. He concluded that the presence of histone was responsible for the nuclear reaction. Saint-Hilaire also showed that nucleohistones, precipitated in the tissues by acetic acid, were dissociable by means of dilute solutions of hydrochloric acid, leaving the histones *in situ*. Variations on this theme are widely employed at the present time.

Especially in the second half of the period we are considering, a great deal of work was done on the nature of protoplasm, particularly by Stöhr (1882), who, as the result of coagulation studies, concluded that a protein substance was present in gastric parietal cells, and by Flemming (1882), Kossel (1882, 1886), Altmann (1886, 1889), Schwarz (1887), and Mann (1890). Flemming (1876) had already described a cement substance holding together the fibrils which compose the loose connective tissue bundles. This he considered to be of mucinous nature. Altmann (in 1889) developed his method of fixation by freezing and drying, which has become an important modern tool of histochemistry in the hands of Gersh and his successors. Others continued to work on the chemical nature of staining and much of their work has modern applications. Among works of particular merit are those of Griesbach (1886), who postulated that tissue dye compounds should have properties differing from those of the free radical ; of Unna (1887), who tried to confirm this ; and of Lilienfeld (1893), who investigated the staining of mucins. Hoyer (1890) demonstrated metachromasia in the cells of the mucous salivary glands with thiazine dyes and, following his discovery, metachromatic methods for mucin became quite popular. Before Hoyer published his paper mucins were usually stained by techniques making use of their strong basophilia. List (1885) was the first to use Bismarck brown for this purpose and the same dye, in alcoholic solution, was used by Hardy and Wesbrook (1895) to stain water-soluble mucoproteins and mucopolysaccharides. In the same year, Heine (1895) made some important observations on the nature of chromatin. He observed that segments of this substance were intensely stained by Millon's reagent and tried, unsuccessfully, to distinguish between nucleoproteins and nucleic acids using mixtures of methyl green and rubin S. Also in 1895, Macallum demonstrated that, after treatment with sulphuric acid, the nuclei were stainable by the usual methods for iron in the tissues. Since this time the question of whether this iron is really present in the nuclei, or adsorbed from elsewhere, has been debated at length.

Enzyme methods of the closing years of the nineteenth century are represented by the contributions of Mall (1891), who investigated the swelling of collagen in various solutions and the action upon it of crude preparations of pepsin and trypsin. Nothing approaching the modern concept of enzymal analysis was achieved at this time. Daddi, in 1896, first used Sudan III for the *in vitro* staining of fat which, after being subsequently ingested by animals, was demonstrated in the tissues by its red colour. Sudan IV was proposed as a fat stain by Michaelis in 1901. This author showed that the staining of fats with Sudan dyes was purely physical, depending on solution of the inert dyes in the fats themselves.

Among methods for revealing inorganic salts in the tissues may be mentioned the techniques of Molisch (1893) who stained tissue-iron by converting it to the red thiocyanate, and of Lilienfeld and Monti (1892), who evolved an ammonium molybdate technique for demonstrating phosphate. This method was modified by Pollacci (1900) and it has been further modified by other workers in the twentieth century. The forerunner of a number of very similar techniques for demonstrating metal salts in the tissues was de Michele's (1891) method for mercury, which he converted to its sulphide by means of H_2S. The method which we still use to demonstrate the presence of calcium in the tissues was described by von Kóssa in 1901 ; even then it was preceded by the more specific " gypsum " method, described by Schujeninoff (1897), which also survives to the present day.

It is probable that this brief account of the history of histochemistry has failed to give the reader a true sense of the continuity of the science from decade to decade. This is at least partly due to the fact that real continuity was lacking. Nevertheless, it is to be hoped that I have been able to show that the science of histochemistry as formulated by Raspail, and the principles expressed by that great man, continued without serious interruption from the 1820's to the twentieth century. When the nineteenth century came to a close, the majority of histologists were occupied in reaping the rich harvest presented by new developments in the art of staining and few had time to spare for histochemistry. The subject therefore remained for the most part in abeyance, though kept alive by a few practitioners until its revival in the 1930's and its establishment on a modern footing as an independent branch of histology by Lison with his great work *Histochimie Animale*. Since his time expansion has been rapid and progress almost equally so, until now, in the second half of the twentieth century it is to be hoped that the majority of histologists would wish to " recognize micro-chemically " some at least of the substances in the tissues with which they deal.

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CHAPTER II

COLD KNIFE AND COLD MICROTOME (CRYOSTAT) METHODS

With the development of an increasing number of techniques for demonstrating enzymes the need for fresh tissue sections has become more important. It is no longer possible to circumvent the difficulties attached to the production of a large number of such sections by using alternative methods involving fixation and, sometimes, embedding. Freeze-drying, the obvious alternative, has now been tested in many laboratories and found wanting in several respects. These will be referred to in Chapter III. The various methods for cutting fresh sections fall into two broad divisions, cold knife methods and cold microtome (cryostat) methods, though there is some degree of overlap between the two.

Cold Knife Methods

Freezing microtomes provided with means for cooling the knife blade with an additional CO_2 jet were introduced into histological technique by Schulze-Brauns (1931a and b, 1932). Commercial models have been available for a number of years and they have been used by many workers (e.g. Wachstein and Meisel, 1953) for the provision of semi-serial sections for histochemical use. Difficulties attending the use of such microtomes in practice have resulted in a search for better means of controlling the large numbers of variable factors which play a part in the production of perfect fresh sections thin enough to allow detailed studies to be made. The various procedures and forms of apparatus which have been developed for this purpose will be discussed below in some detail.

Although the use of standard freezing microtomes with knife-cooling attachments comes under the heading of cold knife methods, it is now customary to use the term to refer to the type of method developed by Adamstone and Taylor (1947, 1948). These authors used a Leitz base sledge microtome with the knife blade cooled continuously by the application of dry ice (solid CO_2), held on the knife surface by retaining fences on either side of the cutting area. With the Adamstone-Taylor technique, as with previous methods using the cold knife principle, curling or rolling of the sections is prevented by gently stroking the section upwards on the knife (with moving knife microtomes) by means of a fine camel-hair brush. The flattened section is lifted from the knife by means of a double-bottomed metal scoop,

containing dry ice in its lower half, and transferred to the surface of a clean glass slide. At the moment melting begins, and the section begins to spread on the slide, the two are immersed in a Coplin jar containing either a fixative or some specific reagent in solution. Adamstone and Taylor stressed particularly the necessity to avoid complete melting on the slide if fine structure was to be preserved, and they described the rupture and distortion of cells which occurred if such precautions were not observed. With this in mind, Adamstone (1951) produced an automatic device which immersed the sections in the chosen fixative at precisely the right moment. With a single operator such a device is almost essential and, as the original authors observed, the whole method is much more efficient if two operators are available. There must be few laboratories, in fact, in which a spare pair of hands cannot be found.

The rapidity with which reagents, including fixatives, can be applied to tissue sections constitutes an important advantage of the Adamstone-Taylor method as does the saving of time by comparison with standard techniques of dehydration and embedding. A severe disadvantage, as pointed out by Taft (1949) is the dependence of the method on atmospheric conditions. Air temperatures over 18° (65° F.) and excessive humidity interfere with its successful performance so that a cool or cold room should be employed if available. Alternatively, I am told, a fine-mesh net containing powdered dry ice may be suspended above the microtome and its operator during cutting and mounting. If a strong cooling effect is required shaking the net by means of a string induces a fine shower of dry ice, but the flow of cool air from the under surface of the net may be found adequate. Taft believed the Adamstone-Taylor method to be best adapted to use in experimental laboratories, but there is no doubt that arrangements can be made in most routine laboratories for the carrying out of some kind of effective cold knife procedure.

The Adamstone-Taylor method has, in fact, been favourably received on the whole and it is now widely used throughout the world. White and Allen (1951), for instance, reported a successful modification of the Bausch and Lomb rotary freezing microtome for cold knife sections. They were able to cut serial sections 6–8 μ thick in almost unbroken series. Personal experience of this excellent microtome has shown that such a performance is well within its capabilities. A most interesting adaptation was described by Shimizu, Kubo and Morikawa (1956), who employed a Schanze sliding microtome, with the knife cooled on either side by blocks of dry ice. In place of the customary camel-hair brush they used a device for preventing the curling of sections (Fig. 1.) based on the principle evolved by Linderstrøm-Lang and Mogensen (1938). The whole microtome stands in a

domestic refrigerator (5 cubic feet) whose front has been replaced by a wooden door through which pass two gloved armholes for the operator and the CO_2 supply to the microtome. Interior illumination is provided and a small viewing window. More recently Thornburg and Mengers (1957) have carried out some important studies on optimum conditions for fresh frozen section cutting, using a Spencer sliding microtome incorporated in a modified kitchen type refrigerator. Their apparatus is shown in Figs. 2 and 3. The provision of environmental control in the manner described above certainly overcomes the chief objection to the method of Adamstone and Taylor, that is to say, its sensitivity to atmospheric conditions.

When it comes to the handling of cold knife sections, there are wide differences of opinion and usage. The two main alternatives are :

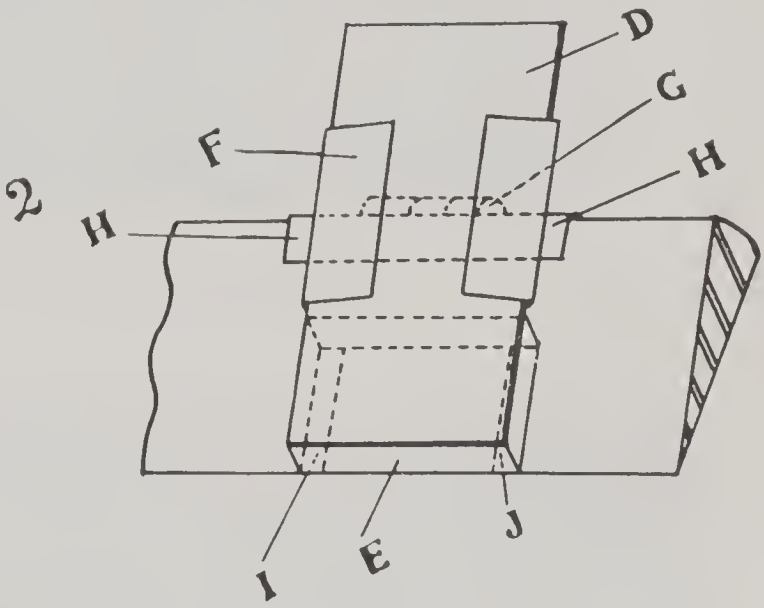

Fig. 1.

D. Glass slide.
E. Cutting edge of knife.
F. Slide holder.
G. Hinge.
H. Hinge clamp.
I.J. Distance pieces.

(1) to pick up the section on a glass slide, prepared or otherwise, or (2) to take up the section with some instrument, cold or otherwise, before transferring it to another situation. Adamstone and Taylor gave the strongest arguments in favour of applying the fixative or other reagent directly to the still frozen sections, as mentioned above. Wachstein and Meisel, on the other hand, found it difficult to fix the still frozen sections to the slides and they therefore transferred them " while thawing " to whatever incubation or staining medium it was intended to employ. Shimizu and his collaborators transferred their sections to slides with a dissecting needle, removed them from the refrigerator, and allowed them to dry at room temperature. Thornburg and Mengers preferred to use the Adamstone technique with an automatic section dropper installed in their refrigerator by the side of the microtome. Many workers, myself included, have been in the habit of carrying cold knife sections directly into incubating media by means of cold section lifters or camel-hair brushes. Further discussion

on the handling of sections may be deferred until after the section on cold microtomes which follows, since the problems involved are essentially the same.

Cold Microtome Methods (Cryostats)

I use the term cold microtome in place of, or as well as, the more customary term cryostat for two reasons. First, the essential difference between the methods of this section and those of the previous one is that the microtome, the knife, and the tissue block are all at the same temperature (— 12 to — 22°). Secondly, the term fits into line with the accepted terms cold knife and cold knife sections, so that one may refer to the cold microtome and cold microtome sections. The alternatives, cryostat and cryostat sections, are perfectly acceptable, but the cryostat (literally a device for maintaining cold, *viz.* a refrigerator) is not primarily concerned with the production of sections. By this definition the cold microtome differs from the cold knife with cold environment (Shimizu *et al*, Thornberg and Mengers) simply in the fact that sections are cut without the provision of cold from outside sources and, unless an attachment of the type described in Appendix 2, p. 784, is fitted, without the provision of a temperature gradient between the knife and the tissues. The cold knife in a cold environment is therefore to be regarded for all practical purposes as a cold microtome.

The first cryostat was developed in Denmark by Linderstrøm-Lang and Mogensen (1938) for their studies in quantitative histochemistry in which alternate frozen sections were taken for chemical estimation and for histology. In the original cabinet cold was maintained by blocks of dry ice, and from this to the provision of refrigerating coils was a simple step. The microtome was modified by the provision of a glass plate in front of the knife which effectively prevented rolling or curling of sections. No real attempt was made to apply the cold microtome technique thus developed by the Danish authors to histochemical techniques in general. In 1951 Coons and his collaborators (Coons, Leduc and Kaplan) made a number of technical improvements in designing a new cryostat of some 6 cubic feet capacity. This model is now made commercially in the United States by the Harris Refrigeration Company of Cambridge, Mass., and in Britain by the Prestcold Company at Cowley, Oxford. The Coons cryostat was originally designed for working temperatures between — 16 and — 18°, though a temperature as low as — 30° can be reached with later models employing more powerful refrigerating machinery. In the sides of the cryostat gloved armholes are provided through which the microtome is operated. The interior is illuminated and provided with a fan (switched off while cutting is in progress) to maintain the circulation of cold air. Below the window is a door giving access to the chamber.

FIG. 2. Domestic refrigerator converted as a microtome chamber with glove holes and lucite window.

FIG. 3. Interior view showing freezing microtome (sliding type) and slide-dropper for instantaneous fixation (Adamstone-Taylor).

[*To face p.* 16.

FIG. 4. Commercial cryostat (Dittes, Heidelberg). A Jung rotary microtome is operated by inserting the hands through side ports. The cutting stroke is externally operated by an assistant.

FIG. 6. Commercial cryostat (Pearse-Slee, London). All controls of the standard Cambridge rocker-type microtome are externally operated.

An excellent Coons-type cryostat is produced in Germany by Dittes (Fig. 4) which has a number of advantages over the original. A standard Jung rotary microtome is used and rusting is avoided by reducing the humidity of the microtome chamber to a low value. The chamber is not intended to be opened and access is through armholes and a small instrument port.

The microtome commonly employed in the United States is a rotary machine of the Minot type but, unless this is specially (and expensively) constructed of stainless steel, it must be removed at frequent intervals, dismantled, cleaned and dried, oiled and reassembled. Microtomes made of brass have also been employed in order to avoid this complication. Normally, rotary microtomes are constructed with such small tolerances that they are acutely sensitive to cold, and

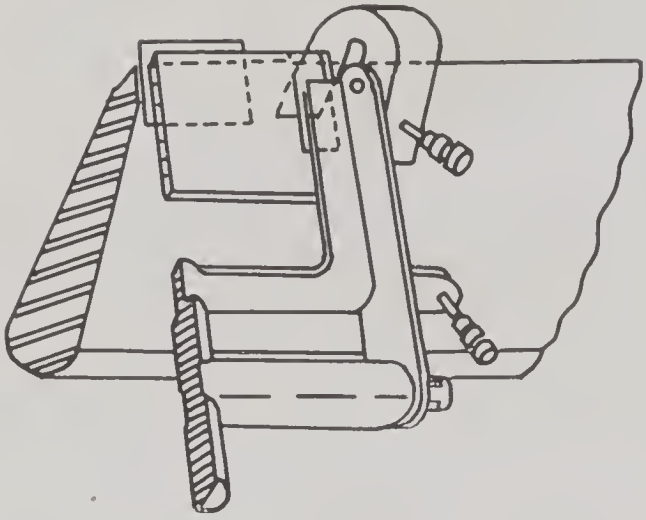

Fig. 5.

stiffening of the whole mechanism is experienced when these microtomes are employed in a cryostat. An essential part of the Coons cold microtome is the ingeniously designed " glass window " which is applied to the knife to prevent curling up of the sections during cutting. The Coons type of anti-roll plate is illustrated in Fig. 5. It includes adjusting screws which allow height, angle and distance from the knife edge to be regulated. A rotating pin allows the window assembly to be swung aside after the section has been cut so that it may be picked up on a cold slide kept in readiness in the cryostat. Correct adjustment of the anti-roll plate is second in importance only to the condition of the knife edge in cutting good thin sections on the cold microtome.

After carrying out a number of experiments with various materials having a low coefficient of friction, in place of the glass microscope slide usually employed, I have found the most suitable material to be polytetrafluoroethylene (Fluon, Teflon). The slight drawback that this material is translucent but not transparent is compensated by increased confidence in the cutting of unblemished sections.

The success of the fluorescent antibody technique (see Chapter VI) and the fact that Coons' excellent cryostat became commercially available was rapidly followed by the extension of cold microtome methods to a wide variety of histochemical techniques. Foremost among these have been enzyme techniques, primarily those for which fresh sections are essential but also some which normally utilize fixed sections prepared in different ways.

Comparison of the cold knife and cold microtome methods for the production of large numbers of thin fresh frozen sections for enzyme histochemistry convinced me that only by means of the second could technical efficiency be raised to the point where blocks from all types of tissues could be successfully cut on demand and processed accordingly. Bearing in mind the ever-present need to apply the techniques of modern enzyme histochemistry to surgical and autopsy specimens, whose time of arrival can neither be controlled nor predicted, a constant running, remotely controlled, cold microtome and cryostat assembly was designed and produced.

The following points were intended to be satisfied :

(1) Low initial cost and constant running with minimum attention.

(2) Instant availability for rapid diagnostic biopsies.

(3) Thermostatic control between — 5 and — 20°.

(4) Minimum temperature below — 20°.

(5) *Automatic* delivery of 8–30 μ *serial* sections directly into incubating media or fixatives.

(6) Production of 4–30 μ *serial* sections, singly or in pairs or triples, for handling on slides or coverslips.

Because of the need for instant availability only two methods of obtaining and maintaining cold could be considered. These were (1) mechanical refrigeration, and (2) CO_2 gas in cylinders. The first was used for maintenance of cold in the microtome chamber, and the second, whenever rapid processing became necessary, for freezing the tissue blocks on their brass holders. The temperature of the chamber is normally kept at — 14 to — 16° by means of a thermostat but, when large numbers of blocks are to be cut, the refrigerating unit can be run continuously to give, at the usual mean summer temperatures in Britain, a minimum temperature of — 35°. The atmosphere in the chamber is kept dry by means of a bag of silica gel (renewed at weekly intervals), and the resulting development of static electricity in the microtome is overcome by means of a chain connecting the microtome to the walls of the chamber. These are earthed through the motor of the refrigerating unit.

The whole apparatus is shown in Fig. 6, and the interior mechanisms and the microtome in Figs. 7 and 8. Easily visible on top of the machine is the resetting control which runs through a tufnol block and

a flexible shaft to the rotating head of the microtome advance mechanism. Just above the handle of the knife, in Fig. 8, is seen the spring-loaded section thickness selector which clicks at every 4 μ advance of the normal selecting lever of the microtome from 0 to 30 μ. External operation of this control is a useful refinement.* Coming in from the left side of the cabinet is seen the flexible shaft for the anti-roll plate control whose knurled knob is invisible on the outer wall of the cabinet.

On the right side of the cabinet in Figs. 7 and 8 is seen the cutting wheel, whose rotary motion is transferred by means of a spring-loaded chain and pulley to the reciprocating lever, visible in Fig. 7, which

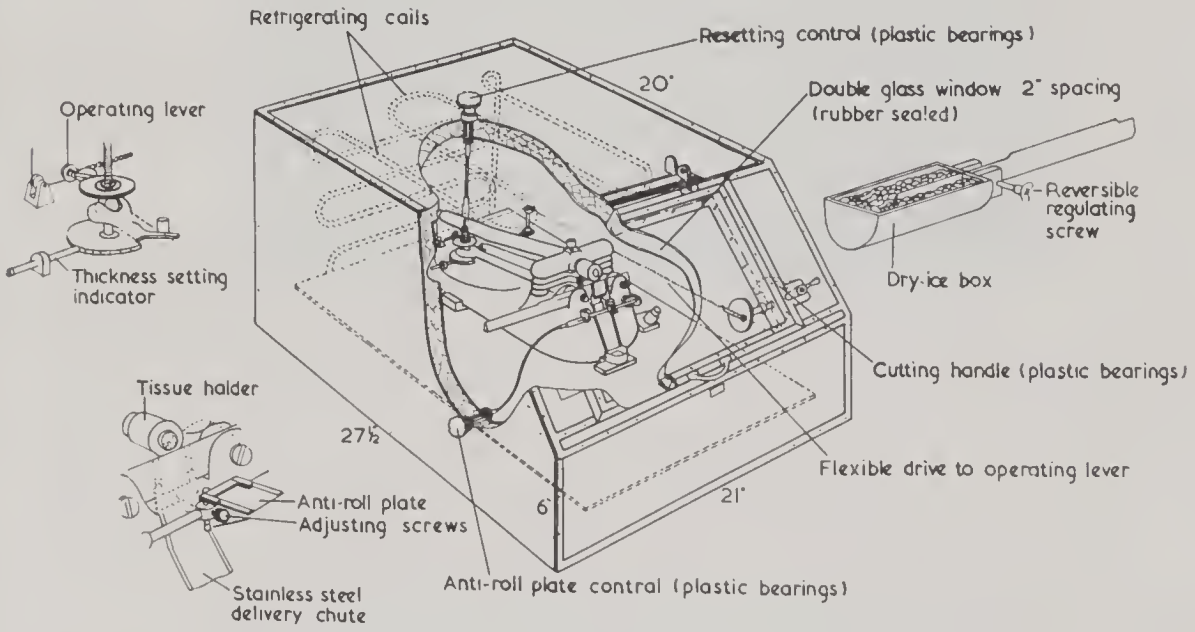

FIG. 7.

normally actuates the advance mechanism of the microtome. The large double glass door gives wide access to microtome and accessories and allows sufficient light to penetrate so that internal lighting is superfluous. It can be thrown back on to its stops for such procedures as changing blocks, but is normally opened only sufficiently to allow removal of sections or containers.

Consideration of Optimal Conditions for Cutting

In their first paper on the analysis of frozen section technique Thornburg and Mengers (1957) pointed out that in frozen tissues water could be regarded as the embedding medium, and sectioning, therefore, as a matter of cutting ice. This conception has obvious limitations. Practical experience leads one to regard the cutting of brain, for instance, much more as a matter of cutting butter. Nevertheless the

* In the commercial models produced by South London Electrical Equipment Co. Ltd., Hither Green Lane, London, S.E.13, and by Bright's Refrigerator Service Ltd., 399 Portobello Road, London, W.10, the thickness selector is remotely controlled.

conception is true enough to form the basis of some interesting theoretical and practical deductions. Thornburg and Mengers found that the optimum values for the temperature of the knife, the chamber and the tissue block varied from tissue to tissue. Cutting was usually possible, however, within a wide range of temperatures.

(1) **Tissue Temperature.** As is the case with ordinary freezing microtomes, when the block temperature falls below — 45° the tissues are brittle and friable and cannot be cut. Between — 40° and — 15° block temperature Thornburg and Mengers could cut thin sections, but they noted resistance during the cutting stroke and some crumbling of sections on the knife. At higher temperatures, up to — 5°, thin serial sections could be obtained.

With isothermic machines (knife and tissue temperature identical) cutting is possible between — 30° and — 10°, but at the higher end of the scale the sections tend to be compressed as they pass between the knife and the anti-roll plate.

(2) **Chamber Temperature.** With the cold environment-cold knife machine chamber temperatures between 0° and — 10° were found best. Below — 10° the quality of the sections began to deteriorate. The working temperature of most Coons-type cryostats is well below this level ; as a rule a constant — 20° is maintained. This is doubtless a necessity on account of the relatively high knife temperature in such cabinets. It is a general rule in frozen section cutting that the higher the knife temperature the lower must be the temperature of the block.

Knife Temperature. In Thornburg and Mengers' cryostat the optimum knife temperature was found to be in the region of — 50° with the tissue block at — 10°. In Coons-type cryostats with the tissues held at about — 20° a similar knife temperature gives good results with most tissues.

In the case of my own cold microtome a dry ice box in the form of a hemicylindrical trough is carried on the knife handle to which it is attached by means of a reversible screw (to allow left-hand or right-hand fitting). This dry ice box is illustrated in Fig. 9. A single charge of pulverized dry ice lasts for about 3 hours (in the early stages it tends to lower the chamber temperature from — 20° down to — 25° or thereabouts) and the temperature of the actual cutting area is varied by sliding the box in and out on the handle. The provision of this attachment gives knife temperatures between — 30° and — 60° which are essential for critical work and if serial sections thinner than 8 μ are to be cut with any degree of certainty.

A Theoretical Analysis

In order to provide some interpretation of their results Thornburg and Mengers produced a model for the cutting process suited to their

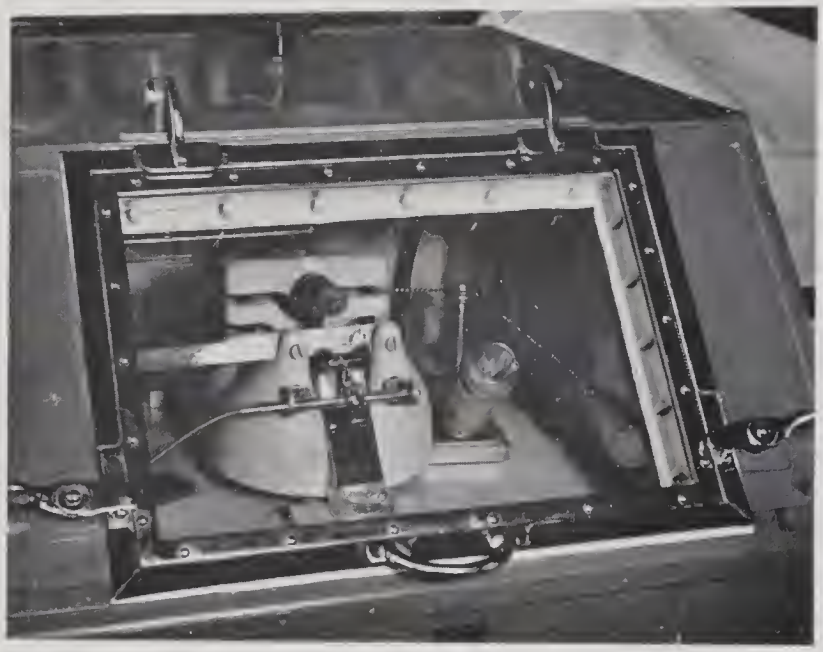

Fig. 8. Interior view of the prototype Pearse cold microtome ; shows various controls and stainless steel chute for delivery of ribbons of sections.

Fig. 9. Stainless steel dry ice box for attachment to Heiffor pattern knives used in Pearse-Slee cold microtome.

[*To face p.* 20.

own cabinet and microtome. An adaptation of this to fit the cold microtome with dry ice accessory for cooling the knife is shown in Fig. 10 below. Making the assumption that fresh-frozen tissue is physically equivalent to ice, Thornburg and Mengers calculated the energy dissipated in cutting a single frozen section. For a block having a cutting resistance of 20 g. per cm. width this is $1{\cdot}96 \times 10^4$ ergs. By converting this figure to calories and dividing by the heat of fusion of ice (73 cals./cm.^{3}) a figure of $6{\cdot}5 \times 10^{-6}$ cm. (650 Å) is obtained for the thickness of the layer of frozen tissue which will be melted by the cutting stroke. The heat generated by the latter can be expected to appear in the region of the cutting edge as a " zone of fusion." Tissues melted in this zone will refreeze as they pass down the two surfaces of

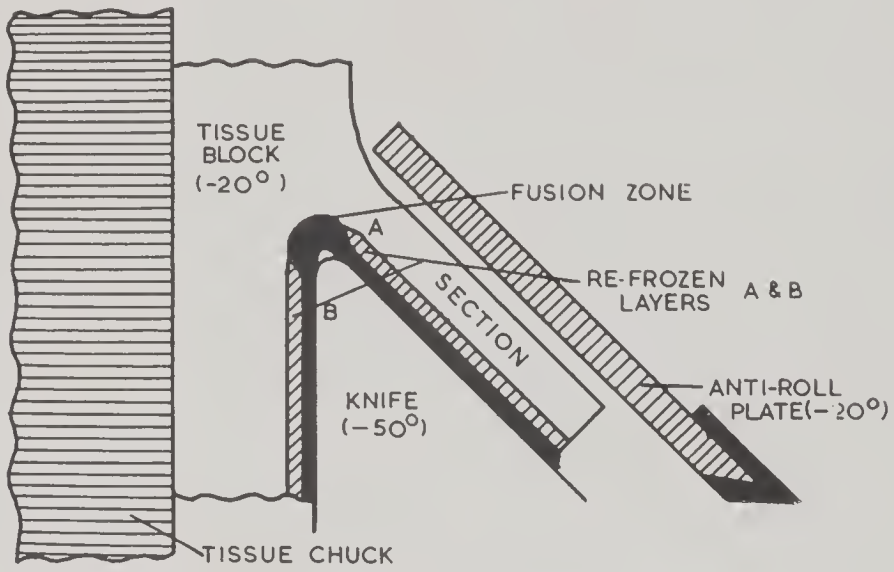

Fig. 10.

the knife, and it is probable that the rate of refreezing at the point A in the diagram is a critical factor for the successful delivery of a perfect section between the knife and the anti-roll plate. A second critical factor here is the rigidity of the section, which must be cold enough or thick enough to withstand frictional resistance to its progress over the knife surface. A clean surface is essential, even a film of condensed vapour is sufficient to increase this resistance beyond the critical point.

The original authors have based a number of deductions upon their model of the cutting process. These are that : (1) the thickness of the melting zone increases as the block and knife temperatures rise ; (2) as the knife temperature is lowered a larger fraction of the energy dissipated in the cutting process is conducted directly into the knife and the thickness of the melting zone is decreased ; (3) as the surface temperature of the block is lowered increased pressures are necessary to maintain the melting zone. Thornburg and Mengers predict on

theoretical grounds that conditions must exist under which sections a small fraction of a micron in thickness can be cut from fresh frozen tissues. They also predict that sections cut under optimal thermal conditions will show a surface artifact due to the melting zone. Optimal thermal conditions for cutting are not necessarily equivalent to optimal conditions for tissue preservation and one must add that, since every tissue possesses its own individual optimum cutting and optimum preservation conditions, the only practicable method of determining these is by experiment. The cutting of perfect fresh frozen sections with modern cold microtomes and cryostats is likely to remain, therefore, a skilled technology.

Handling of Sections after Cutting

With all the cold knife and cold microtome methods described above, handling of the tissue sections after cutting is just as critical as the operations leading up to their production, more so once these have been brought to perfection. A number of different techniques have been evolved by various workers in the field and it must be said at once that no one method is better than all the others. All represent some form of compromise, as will be seen, and the right method for one purpose may well be the wrong one for another.

There are some eight different ways by which cold knife and cold microtome sections can be treated.

(1) Pick up on warm (room temperature) or cold (cryostat temperature) slide or coverslip. Fix by immersion at moment of thawing (Adamstone-Taylor). Alternatively immerse in cold "protective" medium.

(2) Pick up on warm or cold slide or coverslip, thaw, and dry in air.

(3) Pick up on warm or cold slide or coverslip, thaw, dry, and post fix.

(4) Pick up on materials other than glass (e.g. cellulose acetate film, stripping film, etc.).

(5) Transfer or deliver into warm or cold incubating medium (enzyme histochemistry).

(6) Transfer or deliver into warm or cold test reagent solution (especially protein and inorganic histochemistry).

(7) Transfer or deliver into warm or cold fixative.

(8) Freeze dry (for subsequent chemical or biochemical assay).

For the majority of purposes the first three methods are the methods of choice. For histochemical studies on labile enzymes methods 2 or 5 are absolutely necessary. If the enzyme under consideration can withstand fixation, however, methods 1, 3 or 7 may be applied. In the case of soluble enzymes some such procedure is essential unless

the fixative is incorporated in the incubating medium (simultaneous fixation). Freeze drying of fresh frozen sections is normally employed only in quantitative histochemistry.

It is important to realize that none of the methods described above is free from objections and that, as a rule, the choice of method must be dictated by practical rather than theoretical considerations. Complete destruction of cells, described by Adamstone (1951) as inevitable unless immediate fixation is applied, is rather more than usually occurs when sections are allowed to thaw and dry unhindered on the slides. This process, however, produces stretching and shrinking artifacts and I believe it should be avoided whenever possible. It is likely that some kind of protein film formation takes place, analogous to that seen in the case of air-dried blood smears, and this may interfere with the subsequent penetration of reagents although it may also act by preventing the escape of soluble proteins and peptides. Thawing of sections in (on the surface of) buffered media was advocated by Wachstein and Meisel (1953). This method produces stretching artifacts in the case of weakly cohesive tissues such as testis, especially if the sections are thinner than 10 μ. In the case of the majority of mammalian tissues residual artifact visible with the light microscope is very small if the sections are subsequently fixed before further handling by the addition of concentrated fixative to the original medium. There is, however, a considerable loss of soluble protein from such sections which is more easily noticed if the protein happens to have the properties of an enzyme.

Fixation at the moment of thawing is open to the smallest number of theoretical objections although few fixatives are ideal for the purpose if used at room temperature. Advantage may be taken of the rapid penetration afforded by the thin sections to cool the fixatives employed to — 20° or below. Technical details of the maintenance of cryostat and microtome and appropriate methods for handling the sections are described in Appendix 2, p. 783.

Conclusion

No histochemical laboratory can afford to be without the machinery for trouble-free preparation of large numbers of thin fresh frozen sections. This necessitates the provision either of a cold microtome-cryostat unit such as the Coons model or the simpler model which I have described, or of a controlled environment-cold knife microtome such as the Shimizu or Thornburg models. Without the provision of some form of mechanically refrigerated environment the cutting of fresh frozen sections will always be sufficiently time-consuming and exacting to stifle a considerable amount of routine and research investigation at the outset.

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CHAPTER III

FREEZE-DRYING AND FREEZE-SUBSTITUTION

From the histochemical point of view there are many objections to the use of orthodox methods of fixation and embedding and the freeze-drying method was designed with a view to overcoming some of their deficiencies. The chief disadvantages of the ordinary types of fixation are :

(1) Loss of soluble substances, e.g. lipids in fat-solvent fixatives, and proteins, mucoproteins, polysaccharides, and inorganic materials in aqueous fixatives.

(2) Displacement of cell constituents by diffusion. In this category are placed the so-called streaming artifact and other less obvious types of fixation artifact which include the aggregation of small particles into granules, and the clumping of nucleic acids in the nuclei.

(3) Denaturation of proteins, with alteration in their physical properties and those of the various protein complexes.

(4) Chemical alteration of reactive groups, especially of proteins.

(5) Destruction or inactivation of enzymes by (3) and (4).

The basic principle of freeze-drying is the rapid freezing of tissues at about — 160° and their subsequent desiccation *in vacuo* at somewhat higher temperatures until all except a small, tightly-bound fraction of their water content has been removed. By this process of rapid freezing the water in the tissues is converted into ice with the minimum formation of tissue-disrupting ice crystals, which form when material is frozen at higher temperatures. The frozen tissues are then dried *in vacuo* for a variable period, being maintained during this time at between — 35° and — 70°. The dried tissues, allowed to reach room temperature, are vacuum-embedded in paraffin or some other wax and cut in the usual manner.

It must be emphasized from the outset that the process of freeze-drying is not a method of fixation, although it is widely referred to as such. It is a means of preparing tissues for microscopical examination which leaves them in such condition that, for the majority of methods of investigation which are subsequently employed, fixation by one or other of the usual methods is necessary. During the past 10 years the technique of freeze-drying has been used extensively for critical cytochemical investigations and its principles and applications will be discussed at some length therefore. Several types of commercially-produced apparatus for freeze-drying tissues for histochemical

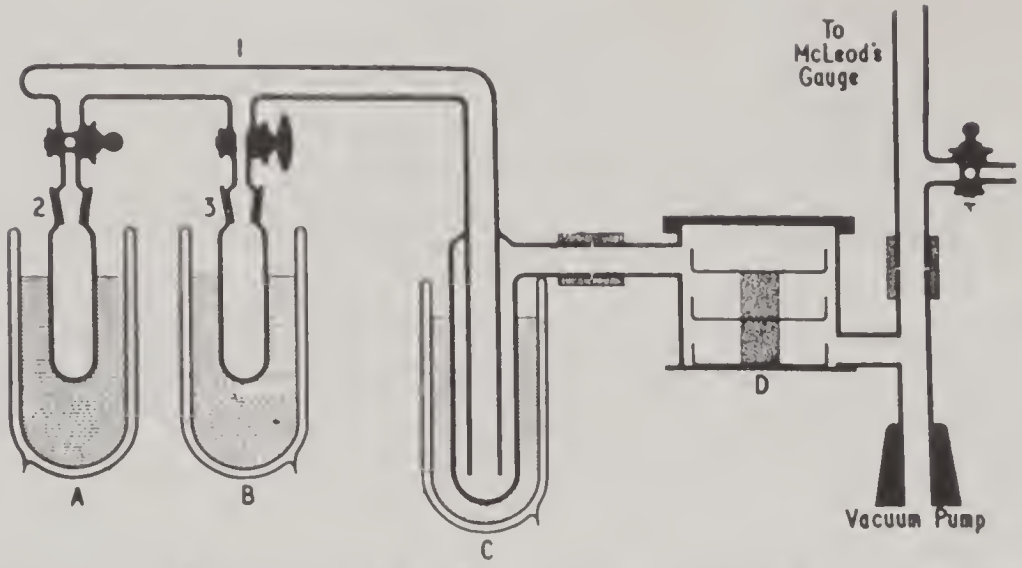

FIG. 11. 1. Manifold. 2 & 3. Drying chambers. A & B. Dewar Flasks containing cooling bath. C. Vapour trap (liquid N_2). D. Vapour trap (P_2O_5). T. Tap (to admit air).

purposes are available in the United States (Phipps & Bird ; Machlett), at least one each in Germany (Leybold) and Italy (Basi Brizio) and a compact and relatively inexpensive model is manufactured in this country by Messrs. W. Edwards and Co. The price of such commercial models varies between £200 and £1,000, and this constitutes an obstruction to the routine application of freeze-drying to histochemical problems. It is possible, however, to make a simple apparatus, which will give perfectly adequate results, for a much smaller expenditure, and such simple types are now being used in laboratories all over the

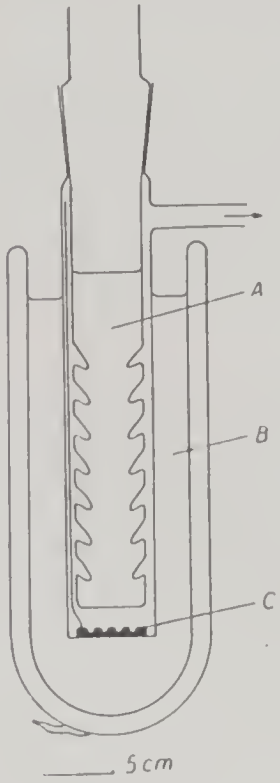

FIG. 12.

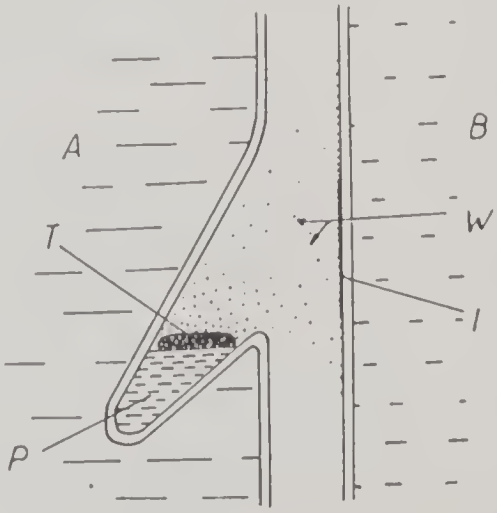

FIG. 13.

world. A very simple and efficient apparatus of this kind, devised by Eränkö (1954a) is shown in Figs. 12, 13 and 14. Those who are completely unfamiliar with the freeze-drying technique would be wise to turn to Appendix 3, at this point, and to make themselves familiar with a simple freeze-drying apparatus and its use. The diagram (Fig. 11) of a simple apparatus may also help in this connection.

The History of Freeze-Drying

According to Mann (1902) the preparation of tissues for microscopical research by drying is the oldest of all methods. He describes Leeuwenhoek as having prepared samples of muscle by this method in 1720, carrying them in his pocket and cutting sections from time to time for microscopical examination. The procedure used by Altmann (1890) was more refined than this and involved the maintenance of small portions of tissue *in vacuo* at — 20°, over sulphuric acid, in a desiccator. After several days the tissues were embedded *in vacuo* in paraffin wax. Mann himself made use of Altmann's method, employing a mixture of solid CO_2 and alcohol to freeze whole animals ; subsequently he broke off small pieces and dried them *in vacuo* at — 30°. Bayliss in 1915 used a similar method but dried his tissues at a slightly lower temperature. The method was of little practical use until Gersh (1932) produced the forerunner of all modern freeze-drying apparatus, and his lead was followed by a large number of investigators. Modifications of the original Gersh apparatus or of the technique for using it have been described by Scott (1933), Goodspeed and Uber (1934), Hoerr (1936), Scott and Williams (1936), Packer and Scott (1942), Gersh (1948), Wang and Grossmann (1949), Mendelow and Hamilton (1950), Stowell (1951), Glick and Malmstrom (1952), Treffenberg (1953), Jensen (1954a), Arcadi and Tessar (1954), Moberger, Lindstrom and Andersson (1954), Lacy and Blundell (1955), Burstone (1956), Caprino (1956) and Naidoo and Pratt (1956). The earlier types of apparatus, and the theory and practice of freeze-drying, have been ably described in an excellent monograph by Neumann (1952). Some of the above modifications will be more fully considered below, following the section on principles and application. An account of the applications of freeze-drying in biological research has been given by Bell (1955) and Cabrini (1955), in an article in Spanish, has reviewed the fundamental principles of the process. The technical side has been considered by Kulenkampff (1956), by Mamulina and Orlova (1955), and also by Hartlieb (1954).

The Principles and Practice of Freeze-Drying

The process of freeze-drying for histochemical purposes can be conveniently considered in four subdivisions. These are : (1) the

initial freezing or, as it is commonly called, quenching ; (2) the subsequent drying ; (3) the processes of embedding, sectioning and mounting ; (4) final treatment before examination.

Quenching. The initial treatment of the tissues by rapid freezing has three main effects. First, it stops all those chemical reactions which are going on in the tissues at the time of their removal from the body which we are accustomed to recognize under the comprehensive term of autolysis. The importance of this effect in critical histochemistry cannot be over-estimated, but the whole benefit of the immediate cessation of chemical reactions in the tissues can only be obtained if material is quenched within a few seconds of the death of the animal from which it is removed. This point is rightly stressed by numerous authors ; Bartelmez (1940), for instance, described the development of particle aggregation and of cytoplasmic vacuolation in pieces of uterine epithelium quenched within 30 seconds of removal. It does not mean, however, as is commonly supposed, that the application of freeze-drying to human biopsy and postmortem material is valueless. This would only be the case if the sole effect of freeze-drying was the prevention of autolysis and sub-mortem change whereas, in fact, this is only a fraction of its function. (By sub-mortem change is meant change of the Bartelmez type whose cause is unknown but which is certainly pre-autolytic.) When the tissues are brought to a solid state, however, the tremendous increase in their viscosity immediately reduces diffusion of substances within them, for practical purposes, to zero. Thus the prevention of sub-mortem change and of diffusion are the two major advantages of the technique.

Its greatest disadvantage is the formation of ice crystals from the unbound water in the cytoplasm of cells and in the tissue spaces, and the subsequent precipitation of materials dissolved in such water at the boundaries of the ice crystals. The latter are of varying size, depending on factors to be discussed below, and it is evident that the smaller they are the more the structure of the freeze-dried sample will reflect the original structure. Conversely, if they are large, resemblance to the original will be small and the artifact will, to all intents and purposes, destroy the structure of the tissue. It is certainly possible to obtain small pieces of tissue in a condition where no ice crystal artifact is visible by ordinary or ultra-violet microscopes. Electron microscopy, however, reveals that ice crystal formation invariably occurs, but it is of such size as to be invisible by other means of examination. Gross ice crystal artifact can be conveniently demonstrated by freezing fixed tissues at $-20°$ and subsequently allowing them to thaw before dehydrating and embedding by the usual techniques. The gross disruption which is observed represents the maximum effect of the process we are considering. If this manœuvre is carried out with unfixed

tissues, fixed only after thawing, all trace of ice crystals is obliterated. A second, but much less important, artifact of quenching is the development of cracks due to the 2 per cent. contraction in volume which accompanies cooling of the block of tissue.

Bell (1952a) has discussed the mechanism of ice crystal formation. It appears that in a fixed volume of a solidifying liquid the number of ice crystals formed is directly proportional to the number of nuclei of crystallization and inversely proportional to the rate of growth of the crystals. In order to achieve the smallest crystal size it is therefore necessary to provide a large number of nuclei and a low rate of growth. Luyet and Gehenio (1938) have shown that the growth of ice crystals in water is reduced by the addition of gelatin. In a 1 per cent. solution the rate of growth is half that in pure water and in a 3 per cent. solution it is 350 times slower. Thus material in solution in water in the tissues slows the rate of ice crystal growth, depending on its concentration and structure. Many substances can entirely inhibit the formation of crystals so that a paracrystalline solid is obtained on cooling. The physical principles of ice crystal growth have been discussed by Meryman (1956). He divides nucleation into two types, homogeneous and heterogeneous, and points out that since the former occurs only with extreme degrees of super-cooling the latter is the usual form. Meryman concludes that crystallization is usually extracellular until rapid rates of cooling are achieved and he considers that the rapidity with which destructive ice crystals can grow renders the thawing process (if this is allowed to occur) as important as freezing. Levitt (1957), writing on the mechanism of freezing in living cells and tissue, substantially disagrees with Meryman's views.

As far as we are concerned, in the practical application of the method, the majority of factors influencing crystal growth are not under control. The most important factor, which is the rate of nucleation (i.e. of appearance of new nuclei of crystallization), can be controlled to some extent, however. It varies with the fall in temperature, but up to a certain critical point the process is very slow. Beyond the critical point it increases rapidly until the material becomes solid. If a system is cooled rapidly to a temperature at which the rate of nucleation is very great, a non-crystalline structure may be formed. In the case of tissues the presence of dissolved materials has the effect of slowing crystal growth so that the point of rapid nucleation may be reached before the tissue becomes solid.

It is evident that in practice, therefore, we have to obtain a rapid rate of cooling. The ability to do this depends on the thermal conductivity of the liquid used for the freezing bath and, if the pieces of tissue are large, it will depend substantially also on their own conductivity. Scott (1933), Hoerr (1936) and Simpson (1941a and b) have

stressed the necessity for using a cooling liquid of high conductivity and at as low a temperature as possible. In his earlier experiments Gersh used liquid air (— 195°) for his quenching bath and although this had a sufficiently low temperature its conductivity was low because of the formation of a layer of vaporized air around the specimen which prevented the transference of heat. In order to overcome this effect Scott (1933) recommended the use of ethyl alcohol cooled to — 100° in place of liquid air, but this method had the disadvantage that ethanol is solid at — 115° and very viscous at higher temperatures in this region. Hoerr (1936) introduced the method of placing the tissues in crude *iso*pentane cooled to about — 165° with liquid nitrogen. Crude *iso*pentane solidifies at — 190° and the point at which it is beginning to become viscous is usually employed. Hoerr also used pentane cooled to — 120°. Various other fluids have been employed for cooling. Emmel (1946) tried *iso*pentane mixed with dry butane to lower the melting-point of the former and Bell (1952a) proposed a mixture of propane (3 vols.) and *iso*pentane (1 vol.) or, alternatively, pure propane, which has its melting-point at — 185°. Some authors, surprisingly, continued to use liquid air directly for quenching (Pease and Baker, 1949), and found no advantage in using *iso*pentane. Simpson (1941b) confirmed the excellence of the *iso*pentane technique but maintained that even by this means the resulting freezing was uneven unless very small pieces of tissue were used. He described three zones in the dried tissue caused by artifact during quenching, an outer zone in which ice crystal formation was minimal and preservation of structure excellent, an intermediate zone in which distortion of structure by large crystals was particularly bad, and an inner zone of better but relatively indifferent preservation. Bell (1952b) recommended Arcton-6 (I.C.I. Ltd), known in the United States as Freon-12, as a safe quenching agent for use with liquid oxygen. This compound, dichlorodifluoromethane, melts at — 158°. I now use it routinely in my own work. Moberger, Lindstrom and Andersson (1954) found pure propane cooled in liquid nitrogen to be the best quenching bath. They advised the avoidance of metal holders for quenching and dropped their tissues directly into the bath. Eränkö (1954b), on the other hand, employed a solid bar of copper, cooled to — 192° with liquid air. He devised a pair of quenching forceps, solid copper discs with an insulating plastic handle, with which the tissue specimens were picked up. The quenched tissues were then transferred to a cool beaker to await transference into the freeze-drying apparatus.

Simpson believed that the structural changes in the intermediate zone were caused, while it was still not solid, by pressure from the hard frozen outer zone. Bell (1952a), on the other hand, considered that it represented a zone in which the temperature of solidification

was too high for rapid nucleation to occur but where the rate of crystal growth was high. In the innermost zone, according to this author, the temperature of solidification is too high for rapid nucleation but also too high for the rate of crystal growth to produce the large artifacts of the intermediate zone. This explanation is more satisfactory than that advanced by Simpson. It is supported by the latter's evidence that the dimensions of the three zones can be varied by altering the temperature of the quenching bath. He found that if this was maintained at — 90°, only the inner zone remained.

According to Simpson the best practice for tissues required for cytological examination is to regulate the size of the sample and the temperature of the quenching bath so as to obtain entirely outer zone preservation. No means exist for calculating these factors for different tissues and the method of trial and error must be employed. Bell (1952a) conducted a series of experiments into the speed of quenching. By placing a thermocouple within a length of mouse intestine and plunging the whole into cooled *iso*pentane he found that the time taken to reach equilibrium with the bath averaged 10 seconds. (Blank for thermocouple—2 seconds.) The critical temperature of water (— 40°) was reached in 4 seconds. Although these figures probably represent the outside limit of those occurring in normal practice, they reveal that a surprisingly long period elapses before quenching is complete.

Drying. Solid materials have a vapour pressure exerted by their molecules in equilibrium with the solid phase. If the partial pressure of a given substance is greater than the equilibrium vapour pressure, at a given temperature, material will pass from the vapour to the solid phase in the process which we call condensation. If, on the other hand, the vapour pressure of the substance is below the equilibrium pressure solid will pass to vapour in the process of evaporation or sublimation. The process of drying the solid block of tissue, with which we were left at the end of the previous stage, is brought about by reducing the partial pressure above the block to a point below the vapour pressure while maintaining the block at a temperature at which it remains solid. Under these circumstances all the ice in the specimen will pass out of it by sublimation.

Theoretically, then, it is only necessary to maintain a low vacuum above the solid specimen for a sufficient period to allow vaporization of its total water content. Packer and Scott (1942) calculated that the volume of water vapour evaporated from a sq. cm. of ice at — 60°, at the pressure of their apparatus (less than 10^{-6} mm. Hg) was 1,600 litres/sec. Since this figure represented at least 100 times the capacity of the pumps they employed, it was evident that some other factor must be concerned. This was supplied by the provision within

the circuit of two vapour traps of the type illustrated in Fig. 11. This figure represents, diagrammatically, the layout of the simple freeze-drying apparatus, with which I started my own work in this field. It suffices still to illustrate the broad principles of the process of freeze-drying. From the drying chambers (2 and 3), which were immersed in Dewar flasks containing a cooling mixture, the manifold (1) passed into a vapour trap (C) immersed in liquid nitrogen, and thence to a further trap (D) containing trays of P_2O_5. This trap was connected to the inlet of a two-stage mechanical pump.

There are three types of vapour trap commonly employed in freeze-drying :

(1) P_2O_5.
(2) Alcohol/CO_2 or acetone/CO_2 mixtures.
(3) Liquid air, O_2 or N_2.

Packer and Scott employed types 1 and 2, but in my own apparatus trap C employed liquid nitrogen. The provision of a trap which continuously removes water from the system is the first essential factor in designing an efficient apparatus for freeze-drying. This trap has the secondary, but far less important, function of protecting the pump against the effects of moisture.

A second critical factor is the temperature at which drying is carried out, that is, roughly, the temperature maintained by the freezing mixture in the Dewar flasks (A and B). Altmann used $-20°$, but suggested that $-30°$ would be better, and Bayliss (1915) dried his tissues at $-35°$. He was the first to draw attention to consideration of the eutectic point (Greek : $\epsilon\upsilon$ easily + $\tau\acute{\eta}\kappa\epsilon\iota\nu$ melt). In general, when a mixture of two liquids is cooled, one of the components will freeze out first, thus concentrating the other. By this process the freezing point is lowered and if cooling is continued the temperature is finally reached where both components freeze out simultaneously and the whole mass then solidifies without a further fall in temperature. This is the eutectic point. The presence of various salts in solution effectively modifies the eutectic point, and Scott (1933) pointed out that the drying temperature originally used by Gersh was not sufficiently low to prevent diffusion of some ions since it was above the eutectic point of many naturally occurring salt systems. Packer and Scott used a temperature below $-54{\cdot}9°$, the eutectic of calcium chloride, since they were particularly interested in the distribution of inorganic materials and since $CaCl_2$ has the lowest eutectic of any naturally occurring salt. It has been pointed out that the eutectic points of complex mixtures, such as those which occur in the tissues we are trying to dry, are very difficult to calculate. The binary eutectic with the lowest melting point is that of $CaCl_2$ and water as

FIG. 14. Simple freeze-drying apparatus designed by Eränkö.

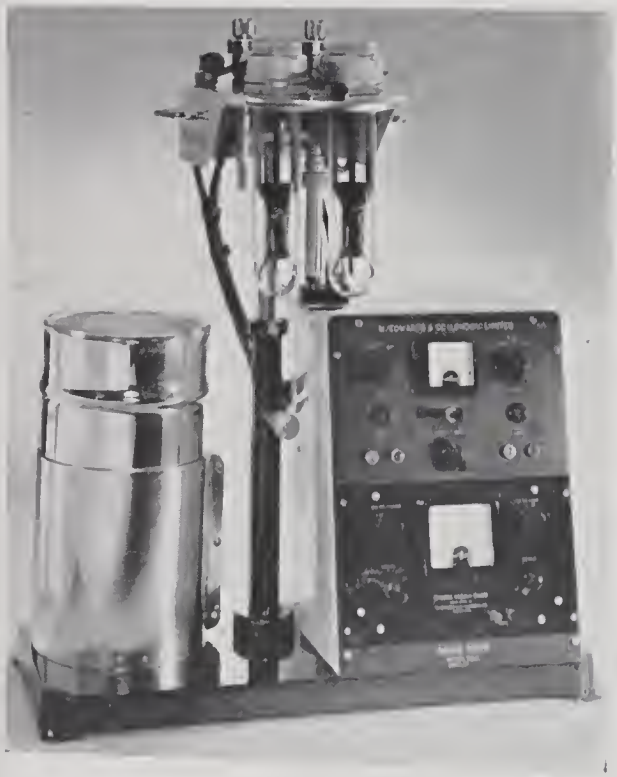

FIG. 16. Commercial freeze-drying apparatus produced in Britain by Edwards Co.

FIG. 17. Freeze-drying apparatus of Naidoo and Pratt.

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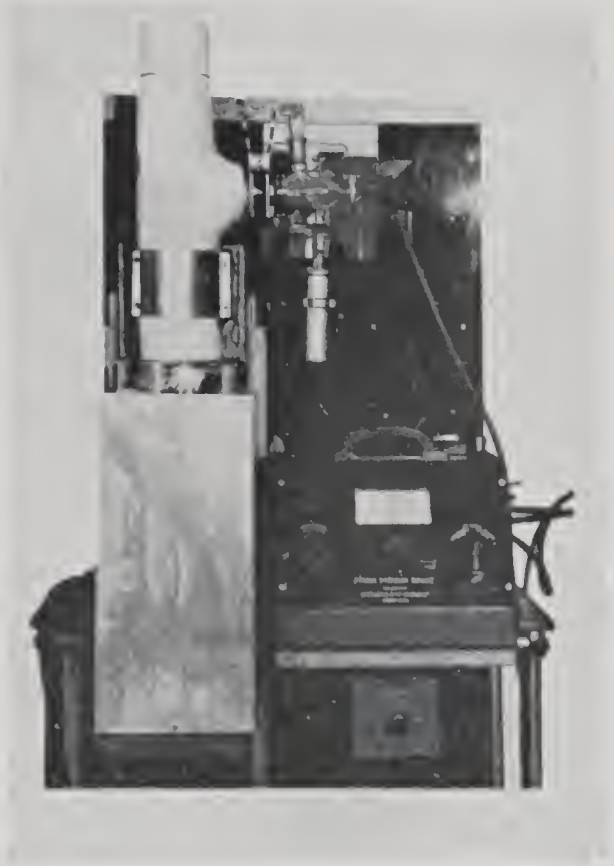

FIG. 18. Large cold-finger type freeze-drying apparatus.

FIG. 19. Another view of apparatus shown in Fig. 18.

FIG. 20. Moving gas stream type of freeze-drying apparatus designed by Jensen.

already mentioned ; tissues, however, contain complex tertiary and quaternary systems with even lower eutectics.

In spite of these theoretical considerations, practical experience suggests that temperatures between — 30° and — 40° are about optimal and, although the majority of workers agree that distinctly better results are obtained at the lower temperature than, say, at — 20°, the eutectic theory would indicate that a still lower temperature would be better. An explanation for this discrepancy is offered by Bell (1952a). It is known that in artificial silica sols, quenched and then allowed to thaw slowly, recrystallization takes place if a critical temperature of — 55° is maintained. Bell suggested that the critical temperature of recrystallization of tissue proteins might be in the region of — 40°, and this would account for the arbitrary selection of this temperature by trial and error. Moberger *et al.* (1954) maintained that temperatures above — 60° were unsafe for the preservation *in situ* of highly diffusible ions and stated that at — 40° morphological changes due to recrystallization appeared.

It is difficult to maintain the tissue block which we are drying at any particular temperature during the whole process, since the evaporation of ice at its surface will lower its temperature compared with that in the centre of the block. All that is possible is to ensure that the temperature does not rise above a given point, and many methods have been used to achieve this. One means of providing the necessary low temperature is by means of a compression-expansion refrigerating plant such as as that employed by Gersh (1932) using liquid ammonia and by Harris, Sloane and King (1950) using methyl chloride. Burstone (1956) has also used a refrigerated box for maintaining his tissues at a low temperature while drying. The majority of workers, however, still use mixtures of solid CO_2 and some organic solvent contained in an insulated vessel surrounding the drying chamber. The original alcohol/CO_2 mixture used by Mann maintained a temperature of — 78° and an acetone/CO_2 mixture is stable at about — 70°. These two are widely used although the temperatures they give are too low for efficient drying in the shortest possible time. This is because the temperature of the specimen controls its vapour pressure and this in turn controls the rate of evaporation. Ice evaporates ten times more slowly at — 60° than at — 40°, at which latter point its vapour pressure is $9{\cdot}66 \times 10^{-2}$ mm. Hg. Mendelow and Hamilton (1950) used a slush of solid CO_2 and ethyl oxalate which is stable at — 40° as long as solid ethyl oxalate remains in the system. This condition can be maintained by infrequent additions of solid CO_2. Other solvents which have been suggested are phenetole (— 34°), nonane (— 53°) and *iso*propyl ether (—60°).

The original Packer and Scott apparatus (Fig. 15) employed a heating

coil to maintain the temperature of the tissue blocks above the temperature of the walls of the container in which they were enclosed. The Stowell apparatus and the apparatus designed in this country and made by W. Edwards and Co. both use the same principle. With most of the older devices of this type, whether they employ a mechanical pump alone or in conjunction with a mercury or oil-vapour pump, the average drying time was in the region of 2–3 days at — 40°, although for some tissues 24 hours was sufficient. With my own original apparatus, illustrated in Fig. 11, a routine drying time of 48 hours was used and the temperature of drying varied between — 70° and — 30°, being usually between — 50° and — 40°. These prolonged drying times were considered by Bell (1952a) to be due to the fact that an apparatus designed with a long length of manifold between drying chamber and vapour trap fails to take into account a third factor in drying, the relation between the distance travelled by water molecules from tissue to condenser, and the so-called mean free path.

Considerations of the Mean Free Path. This expression is used to indicate the distance which any molecule in the gas-containing space of the drying chamber and manifold can travel before it hits another molecule. As such, the mean free path is dependent on the degree of vacuum in the apparatus. If the latter is designed so that its cold condensing surface (the vapour trap) is not further distant from the specimen than the mean free path, molecules of water leaving the tissues will travel to the condenser without colliding with one another. Obviously there must be no bends between the two points or collision will be inevitable. Whether a molecule of water which strikes the condenser rebounds or adheres depends on the temperature of its surface ; the lower the temperature the greater will be the proportion of water molecules trapped. As the distance between the tissue and the condenser is increased the vacuum must similarly be increased if the former is not to exceed the mean free path. The approximate mean free path for a given operating pressure can be calculated, and at $10^{-2.2}$ mm. Hg it is about 5 mm. At 10^{-3} mm. Hg (1 micron) it reaches 50 mm. To keep within the bounds of practicability it is, therefore, necessary to produce an apparatus in which the operating pressure in the drying chamber (not at the pump end of the manifold) is no higher than 10^{-3} mm. Hg and in which the distance between the specimen and the condensing surface is less than 50 mm. It is also necessary that molecules of water which hit the condensing surface should not rebound and this condition is apparently satisfied when the temperature of the latter is maintained at — 185° by means of liquid N_2, or even at higher temperatures.

Many workers found that a convenient arrangement for satisfying these conditions was the provision of a " cold finger," of plain or

silvered glass, which contained liquid N_2 and which was introduced into the drying chamber so that the distance between its tip and the specimen was less than 50 mm. The apparatus described by Mendelow and Hamilton (1950), in which a " cold finger " was provided in the shape of a round-bottomed flask, is illustrated in Fig. 15. These authors used an aluminium desiccator as their drying chamber and I experimented with a double-walled brass vessel of similar dimensions in which the outer space contained the ethyl oxalate/CO_2 mixture. While the use of vessels of this sort is fairly satisfactory, the advantages of metal over glass are scarcely so great as claimed by Mendelow and Hamilton, and the difficulty of maintaining a temperature of — 40° is increased in the case of these large chambers. In 1951, therefore, I used a simpler form of " cold finger " inserted into the original glass drying chambers. This modification is illustrated diagrammatically in Fig. 15, upper right: the " cold finger " was introduced through the top of the drying tube and the manifold was connected instead to the side of the vessel. These modified tubes could still be immersed in the original Dewar flasks. They suffered from the disadvantage that the volume of liquid N_2 which they contained was small and had to be replenished every $1\frac{1}{2}$ hours. With an apparatus of this sort the average drying time for small blocks was reduced to 12–24 hours.

An alternative arrangement which also allowed rapid drying and which avoided the complications due to the " cold finger " was the apparatus designed by Stowell (1951), and shown diagrammatically in

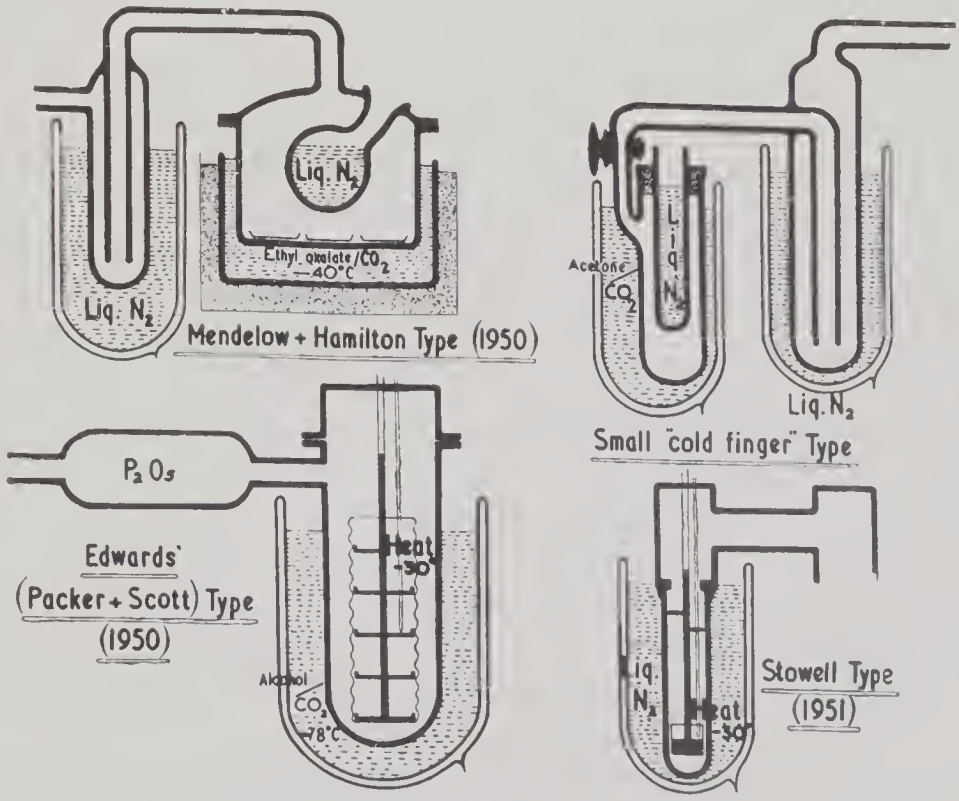

FIG. 15.

Fig. 15. In this apparatus the condensing surface was provided by immersing the vessel containing the specimens in a Dewar flask of liquid N_2. The specimens themselves were heated to any desired temperature (Stowell recommended — 30°) by means of a heater in their support, and this temperature was indicated by means of a thermocouple and potentiometer. The author stated that with this apparatus the time between cutting the original material and the sectioning of the embedded specimens need not exceed 5 hours. The Edwards Tissue Dryer, Model TD 2 (Fig. 16, p. 32), is a particularly efficient apparatus, of the Stowell type, in which the Dewar flask can be filled with liquid oxygen or nitrogen in place of the acetone-CO_2 mixture shown in Fig. 15. It employs a two-stage mechanical pump only and achieves a vacuum in the region of 5 μ Hg.

The simple freeze-dryer devised by Eränkö (1954a) was intended for use in two different ways. If frozen sections were to be dried they were placed in the holder C (Fig. 12, p. 26) and the inner tube A was then filled with liquid air. The Dewar flask B contained an ethyl oxalate-CO_2 mixture (— 40°). Tissue pieces, on the other hand, were placed in the recesses of the inner tube (A, Figs. 12 and 13), which in this case contained the ethyl oxalate mixture while the Dewar flask, B, contained liquid air. Thus in the first instance the vapour trap was constituted by the cold finger, and in the second instance by the walls of the outer tube. Pieces of tissue could of course be dried on the holders, as in the case of frozen sections, and this practice conforms with the accepted one in most establishments. The drying time with this apparatus is 24–48 hours.

Another simple and effective dryer is the one designed by Naidoo and Pratt (1956, 1957) and used especially for the preparation of brain tissues. In this machine (Fig. 17, p. 32) frozen brain slices 1–2 mm. thick are dried in tubes arranged radially around a P_2O_5 trap. The tubes are immersed in vacuum flasks containing a mixture of liquid and solid ethyl phenyl ether (phenetole) which is kept at — 34° by adding solid CO_2 at intervals. A feature of the apparatus is that each tube has a diaphragm valve which allows its isolation from the manifold and a detachable Pirani head (see below). Dryness of the specimens is estimated by closing the valve to isolate the relevant tube and measuring the vacuum therein by means of a Pirani gauge. Drying has been found to be adequate when the reading has remained below 50 μm. Hg/min./g. fresh tissue for 12 hours. Each tube contains degassed paraffin wax so that the tissues can be infiltrated *in situ* under vacuum as soon as they are found to be dry. This apparatus also requires a long drying time of 2–5 days.

When, for any reason, rapid drying is required a more complicated type of apparatus must be employed. The most recent apparatus of

Glick and Bloom (1956) and my own current apparatus (Figs. 18 and 19, p. 33) are similar in that they employ two liquid O_2 or N_2 traps. The near trap is in the form of a cold finger having a large capacity (5 litres, Glick and Bloom ; 1 litre in my own), while the far trap is of conventional type. It must be emphasized that the only advantage which these machines have over the simpler types is that they are capable of much faster drying times. This may be important if radioactive isotopes having short half-lives are being employed.

Practical Considerations for Cold Finger Types of Dryer. On the basis of theoretical considerations Stephenson (1953, 1954), Gersh and Stevenson (1954), and Freed (1955) concluded that the lower pressures obtainable with diffusion pumps did not, in fact, improve performance, and they believed that the physical requirements of a freeze-drying apparatus were not critical. Glick and Bloom (1956) are in substantial agreement with these ideas. My own view is that, provided the apparatus is free from leaks, that the near and far traps contain liquid O_2 or N_2 and that the *critical distance* of the near trap (cold finger) is exceeded, then diffusion pumps are unnecessary. They remain, however, an excellent insurance against the development of leaks during the drying run. With my own apparatus the critical distance of the cold finger has been experimentally determined to be about 3·5 cm. This is the distance between cold finger and tissues at which the latter begin to be cooled by removal of heat to the surface of the finger to such an extent that the drying time becomes excessively prolonged.

Moberger *et al.* (1954) divided the process of drying into an initial 6–12 hours with both near and far traps full of liquid N_2, and a final period, with the Dewar flask (acetone-CO_2, — 60°) removed, in which the tissues are allowed to reach room temperature and the near trap is empty. Glick and Bloom (1956) employed a similar manœuvre, and I usually run an initial stage of 12–16 hours, by which time the near trap is exhausted, before raising the tissues to room temperature for a short period of final drying.

The above considerations, and the successful use by so many workers of dryers which in theory should be grossly inefficient, make it clear that the theoretical conceptions of *mean free path* and *molecular distillation* (Butler and Bell, 1953) are less important from the practical point of view than was formerly supposed. Provided that the tissues are not kept at too low a temperature (below — 55°), pressures of about 10^{-2} mm. Hg from a two-stage mechanical pump will give adequate drying. If rapid drying is required then liquid N_2 or O_2 traps are essential, and with near and far traps both functioning the drying of small blocks of most tissues should not take longer than 10–12 hours. Glick and Bloom (1956) found, for instance, that if 10

hours' drying under low vacuum was sufficient to dry tissues at — 45° to — 50° then 16 hours was required to dry the same tissues at — 78°. With their newest apparatus having a 5 litre capacity cold finger they reported, however, that 6 hours under low vacuum was sufficient to dry tissues at — 78°. These improved drying times were attributed to increased efficiency of the cold finger. This explanation may be correct, but in my experience the temperature of the far trap has been found to affect drying times much more than the temperature, and therefore the efficiency, of the cold finger.

Moving-Air Types of Freeze-Dryer. A number of workers (e.g. Treffenberg, 1953) have suggested or used a different principle of drying in which a stream of cold gas (air or N_2) passes continuously over the tissue, which is maintained in a moderate vacuum. Jensen (1954a and b) introduced a practical dryer employing this principle after finding that the drying times for most plant tissues, using a conventional cold finger dryer, were excessively long. Modifications of the original instrument have resulted in the production of a simple design which is especially suitable for the drying of plant tissues. Jensen's apparatus, which is illustrated in Fig. 20, p. 33, consists essentially of a glass drying chamber surrounded by a coil of glass tubing (centre), a desiccant column and a vacuum gauge. The tissues are placed on a fritted glass plate and the chamber is sealed with an "O" ring. Nitrogen from a cylinder is cooled to — 30 to — 40° by the methyl cellosolve-CO_2 mixture in the Dewar flask and drawn over the tissues by means of a two-stage mechanical vacuum pump at about 1·5 cm./Hg pressure. The pump is protected from the effects of moisture by the provision of a column of desiccant ($CaCl_2$) inserted into the vacuum line.

With the apparatus shown in Fig. 20 it is possible to dry 1 mm. sections of *Vicia faba* epicotyl in 4 hours. Root tips require 12–18 hours depending on the size of the tip, while developing stem tips of *Xanthium sp.* dry well in 8–10 hours. Nitrogen was substituted for the air which was used in an earlier apparatus because Jensen and Kavaljian (1957) found that, in the presence of molecular oxygen, easily oxidizable substances such as ascorbic acid could undergo oxidation in spite of the low working temperature in the apparatus.

Mechanism of Drying with Moving-Air Dryers. It is necessary to enquire into the factors which contribute to the efficiency of moving-air designs. It appears that the most important effect of the stream of cold gas at — 30° to — 40° is to maintain the temperature of the tissues at this level during the earlier stages of drying. A second effect, perhaps equally important, is the rapid transport of water molecules away from the surface of the tissues. Jensen and Kavaljian (1957) have shown that a partial vacuum is essential. If the stream of gas is

passed over the cold tissues at a positive pressure some drying occurs but not more than 40–60 per cent. of their water can be removed, however long drying is continued.

Measurement of the Working Pressure. Various types of vacuum gauge have been employed in connection with freeze-drying, the two most popular being the McLeod and the Pirani types. The former measures the volume of gases left in a specified volume of the system by compressing them in a side arm, while the latter is a hot-wire gauge. The Pirani gauge is sensitive to water vapour pressure, but the McLeod gauge is not, and therefore the latter cannot be used to estimate the level to which drying has progressed. Ionization gauges of course give higher accuracy, but expensive additional apparatus is necessary which makes them, in most cases, an expensive luxury. If the electronic part of the gauge can be borrowed it is sensible to design an ionization gauge head which can be fitted in place of the Pirani head as an occasional check on the latter. This has been done in the case of my own apparatus. All types of gauge can be used to detect leaks in the apparatus but these are as conveniently estimated by means of the simple and inexpensive Tesla sparking coil. If a spark from this is directed on the glass wall of the system (it cannot be used if the apparatus is entirely of metal) a coloured glow indicates a gas discharge unless the vacuum is better than 10^{-3} mm. Hg when only a greenish fluorescence is visible on the glass. The Tesla coil can also be used to indicate the exact site of a leak in glass tubing, and in glass-rubber or glass-wax joints.

Detection of the Endpoint of Drying. Hoerr (1936), using a McLeod gauge, claimed that the completion of the drying process was indicated by a fall in indicated vacuum from ·002 to ·0001 mm. Hg. I have observed a similar fall but consider that it precedes the true dry-point by a long interval. It seems probable that Hoerr's pressure measurements refer only to extraction of air from the system, which of course may be complete before the tissue is dry, although in his case it appeared to be a suitable indication of the dry point. Packer and Scott (1942) used two Pirani gauges, one at either end of the vacuum line, and they claimed that an initial difference between the readings of the two fell to zero when drying was complete. Naidoo and Pratt (1956, 1957), as mentioned above, use the stability of the vacuum when cut off from the pump as an accurate indicator of the endpoint of drying. It can also be decided by the method of trial and error, roughly, by observing that bubbling does not occur when the specimen is placed in paraffin wax in the vacuum-embedding bath and, more accurately, by microscopical examination of sections. Glick and Bloom, for instance, find that if bubbling goes on for 20 minutes the result is bad, but that if no bubbling occurs the result is perfect. In between

these times there are numerous grades, but 1 or 2 minutes' bubbling is usually acceptable. The expression bubbling refers, of course, to the stream of bubbles which rises to the surface after the tissue has sunk in the wax. Initially a slight foam may surround the block and prevent it from sinking. This has no significance.

Only one unexceptionable method for estimating the drying point has been evolved and this is largely of theoretical interest. Jansen (1954) incorporated in his apparatus a torsion balance by which the process of drying could be followed until the endpoint, indicated by constant weight, was reached. Below is a graph of the type obtained by Jansen in his experiments.

It shows that a constant weight is reached in 36 hours with only small

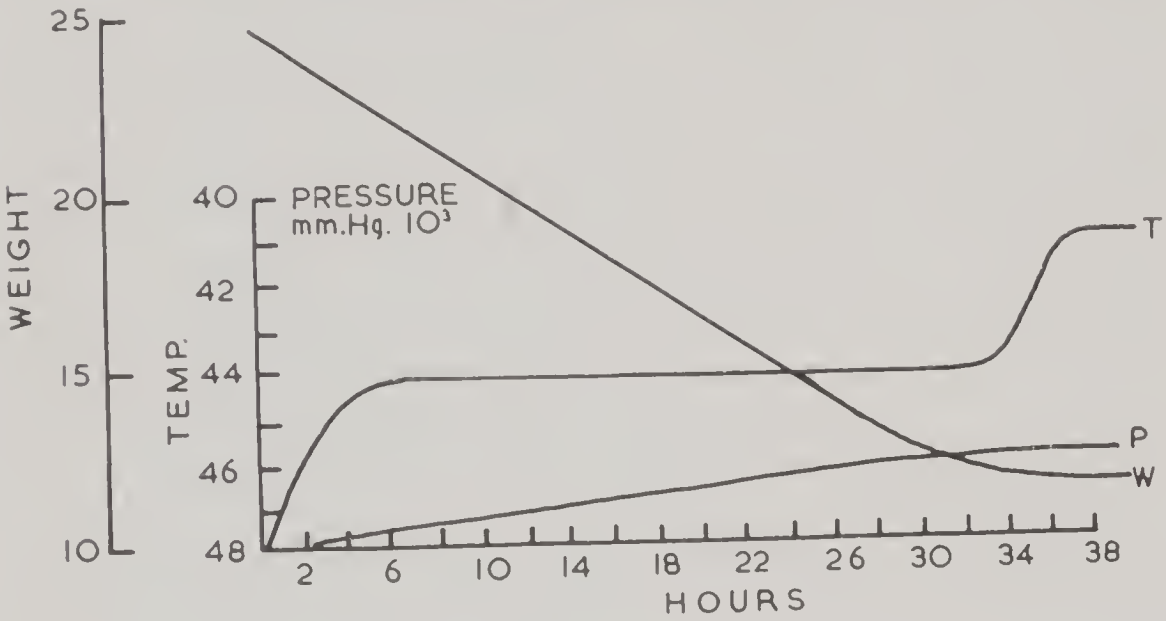

Fig. 21.

pressure variations (measured with the pumps turned off) between 10 and 12 microns. Temperature variations are simultaneously recorded. Jansen concluded that it was difficult to prevent the specimen from cooling too much by withdrawal of the latent heat of evaporation, and that for an adequate heat supply by radiation the object should be surrounded completely [*sic*] by the radiating surface. Alternatively, Jansen suggested fitting the drying tube with a heat-conducting gas at an appropriate pressure. An advantage which Stowell types of dryer possess over the other types, of course, is that tissue temperature is recorded continuously and can be corrected by the operator at all stages in the drying process.

Jansen objects to Gersh's formula relating the drying rate inversely to the square of shell thickness. He maintains that the slope of the weight and time curve (Fig. 21) does not suggest such a relationship.

Embedding, Sectioning and Mounting. One is apt to assume (and

in my opinion most accounts of the technique of freeze-drying encourage this belief) that the quenching and drying stages of the technique are the only important ones. This is not true. Within wide limits the technique of the earlier stages can be varied without important changes in the end result. But all the artifacts which the method seeks to avoid can be introduced with the greatest ease by incorrect handling in the final stages, and to the histochemist, once he has built up (or bought) his apparatus, these stages are the real trial.

For most purposes it is necessary and usual to embed the dried specimens in paraffin wax. At the end of the previous stage we were left with an almost completely water-free specimen at a temperature of $-40°$ and at 10^{-3} mm. Hg pressure. There are two principal methods for embedding this in paraffin. In the first, thoroughly degassed paraffin wax (M.P. about 56°) is placed at the bottom of the drying chamber before the process of freeze-drying begins. When drying is considered to be complete the cooling bath is removed and the drying chamber allowed to reach room temperature. It is then surrounded by a jacket or water-bath at 60° and, at the point when the wax begins to melt, dry air is allowed into the system and the pumps are turned off. By this method there is no exposure of the tissue to moist atmospheric air. In the second method the drying chamber is again allowed to rise slowly to room temperature, air is admitted, and the pumps are stopped. The specimens are then transferred into an ordinary vacuum-embedding bath containing melted paraffin wax. The short exposure to moist atmospheric air does not appear to have any harmful effect, but the practice, employed by some earlier authors, of transferring the cold tissues (at $-40°$) directly into the wax bath at 56° caused appreciable shrinkage.

As an alternative, embedding may be carried out in a water-soluble wax such as carbowax placed, as in the first method outlined above, in the bottom of the drying chamber. Heating may be avoided altogether if the dried tissue block is infiltrated with celloidin, either in the usual alcohol-ether solvent or, preferably, in methyl benzoate, but it is better to follow celloidin by paraffin in a complete double embedding procedure. Simpson (1941b) "infiltrated" the dried tissue blocks for one week with absolute alcohol in order to remove the last traces of water and to reduce the subsequent shrinkage in paraffin. This practice is undesirable since the alcohol itself causes shrinkage, and distortion, by removal of soluble substances. Simpson's cold methyl cellosolve method of freeze-substitution, referred to below and in Appendix 3, is far less objectionable.

Even if the time of paraffin embedding is kept below 10 minutes, as it should be, there is no doubt that the process causes both diffusion of lipid and non-polar substances and also a variable amount of shrink-

age. Goodspeed and Uber (1934) treated their dried tissues with butyl alcohol for a short period before embedding and considered that penetration of the wax was greatly improved. The importance of complete infiltration, which is prevented by the fact that the non-polar wax cannot wet the hydrophilic tissue interfaces, is probably overestimated. It is only necessary to obtain such a degree of infiltration as will afford a measure of support to the tissue during cutting.

Hoerr (1936) avoided the use of all embedding media by cutting freehand sections of the dried tissues, but this does not recommend itself as a practicable proposition for routine use. The ability of freeze-dried tissues to become rehydrated is illustrated by some experiments by this author conducted on such unembedded sections. He showed that if these were cleared in liquid paraffin many irregular vacuoles, due to ice-crystal formation, were visible. Similar sections exposed to 1 per cent. aqueous toluidine blue for 30 seconds (the standard method which he happened to be employing) before mounting showed no such vacuoles. These findings were explained as indicating an uptake of water by the hygroscopic tissue proteins. If a ribbon of paraffin-embedded freeze-dried sections is left on the bench for a short period complete dissolution occurs by absorption of moisture from the atmosphere. To avoid these changes freeze-dried paraffin-embedded sections, after mounting, are usually subjected to some process in which their proteins are denatured by one or other of the common fixatives. If it is desired to keep freeze-dried material for a long period this can be done most conveniently at the unembedded or embedded block stage by storage in trays in a desiccator over $CaCl_2$. Mounted sections on slides may also be preserved for long periods by this method, preferably in the cold room, and in the dark. Such sections are instantly available for any investigations which may be required. Certain unavoidable alterations may occur during storage, however. Bensley and Hoerr (1934), for instance, found that in sections stored at room temperature alterations in the solubility of the tissue proteins occurred.

Sectioning of carbowax embedded material is sometimes carried out on the freezing microtome. More usually such blocks, and those embedded in paraffin or double-embedded in celloidin and paraffin, are cut on an ordinary microtome. Sections 8–10 μ thick are easily handled but below this thickness increasing difficulty in mounting may be experienced on account of disintegration. Floating out on water is out of the question ; with carbowax complete solution occurs and with the other embedding materials partial solution of tissue components. A number of alternate procedures have therefore been employed. Most commonly the floating out process is avoided entirely

and single sections are applied in the dry state to warm, egg-albuminized, slides and flattened by pressure from a finger. Dry mounting on cold slides is not usually favoured. Mendelow and Hamilton (1950) introduced the useful technique of painting a thick film of melted paraffin on to the exposed face of the block between cutting each section, so that each section is backed by a thick supporting layer of paraffin. The sections are then placed cut face downwards on cold slides and secured by gentle pressure. The floating-out methods used for freeze-dried sections fall into two categories, those which avoid, and those which involve, fixation of tissue components. In the first category are methods using mercury or light petroleum (petroleum ether). The use of mercury, warmed to about 45°, was suggested by Danielli and first used by Harris, Sloane and King (1950). Sections floated out on this medium flatten well and are easily picked up by allowing the coated side of an albuminized slide to come into contact with them. If light petroleum is used the sections are instantaneously deparaffinized and simultaneously subjected to severe changes by the removal of lipids. Even if the latter are regarded as unimportant, this method should not be employed.

In the second category are methods using 70 per cent. alcohol, 10 per cent. formalin and other liquid fixatives. Sections floated out on these are, of course, subjected to fixation as well as flattening. The use of 70 per cent. alcohol has nothing particular to recommend it, but, if preservation of lipids is particularly desired, 10 per cent. formalin or formol-calcium mixtures may be employed. If paraffin wax is used for embedding, however, its subsequent removal will remove a large proportion of the lipids present in the section. Carbowax sections floated out on formol-calcium mixtures, or on a formol-diethylene glycol mixture such as that described by Rinehart and Abu'l-Haj (1951) (Appendix 1), should be ideal for lipid studies by histochemical techniques of the type described in Chapter XI.

If we now suppose that the material quenched in Section 1, dried in Section 2, and embedded, cut and dry-mounted in Section 3, has reached us in the form of a 6–8 μ section on an albuminized slide with the wax still *in situ*, there remains only the final treatment before it can be brought to the microscope for examination.

Final Treatment before Microscopy. This depends to a large extent on what particular component of the section we intend to examine, and by what means. The various possibilities can be conveniently considered under four headings :

(1) Examination of unfixed material, in inert media, by physical methods.

(2) Examination of unfixed material by micro-incineration or by treatment with buffer extractions, etc.

(3) Examination of suitably fixed material by conventional histological and histochemical techniques.

(4) Examination by autoradiographic techniques.

Examination in Inert Media. Unfixed freeze-dried tissues are particularly suitable for microscopy by phase contrast, and by polarized, ultra-violet or dark-field illumination. Sections can be prepared by freeze-drying for examination by any of these techniques with the minimum of chemical and physical disturbance to tissue components, and their use in histochemistry in combination with freeze-dried material has greatly increased in the past few years. It has been customary for many years to mount sections in glycerine before examination in polarized or ultra-violet light, or by dark-field techniques, and the use of this medium has been extended to freeze-dried sections. It has been applied either after removal of the wax or leaving the wax *in situ*. Although it is an excellent mountant from the physical point of view, since it reduces the amount of scattering of light at refractive index boundaries, it is not so suitable from the histochemical point of view. Many substances in the tissues are soluble in glycerine and those which are not, such as the majority of proteins, undergo marked swelling and consequent distortion. An alternative mountant, much used for studies on tissue fluorescence, is liquid paraffin. This removes the paraffin wax and is sufficiently inert not to alter the tissue proteins appreciably. Still better results are obtained by mounting directly in nonane, as suggested by Bell (1952a), which also removes the wax. This method is particularly suitable for phase contrast microscopy. Mendelow and Hamilton (1950) described a method of mounting in a synthetic medium such as clarite after removal of wax with light petroleum. If the latter is used for removing the wax, care must be taken to avoid drying before application of the clarite or large numbers of air bubbles will remain trapped in the section. The method is more easily carried out with xylene. For examination of lipids by polarized light I have found it best to use thick carbowax-embedded sections taken quickly through water and mounted in Karo corn syrup.

Examinations after Extractions, etc. The fact that the proteins of freeze-dried sections remain almost entirely undenatured makes it possible for their solubility in various buffers to be determined. In this type of technique, which was employed by Catchpole (1949) on the pituitary mucoproteins and by Gersh (1949) on thyroid colloid, a series of sections is incubated in equimolar solutions of the buffer at different pH levels. After a suitable period of incubation the sections are fixed in alcohol or formalin and the residual protein is stained either by using an acid dye or by the Millon or other specific reactions for protein. An estimate is then made of the point at which the least

extraction of the material under consideration has occurred and this point of minimum solubility is taken as the isoelectric point. Other extractive techniques for proteins are usually performed on sections in which these have been precipitated and denatured but the lipid extraction techniques (see Chapter XI and Appendix 11) can be performed on unfixed material. Many of these techniques will denature and fix the proteins at the same time as they extract the lipids and it must not be assumed, even with non-fixing extractives such as pyridine, that no alteration in the remaining tissues has occurred. There is evidence (Lovern, 1955) that freeze-drying itself may denature some lipoproteins. He states that β-lipoprotein, for instance, is denatured when its water of hydration, which binds lipid and polypeptide together, is removed by any method. Caspary and Kekwick (1957) found an increase in the sedimentation constant of freeze-dried fibrinogen, and Mayersbach (1957) has observed gross differences in the solubilities of normal and freeze-dried proteins after alcohol precipitation. These observations all suggest the need for caution in the interpretation of the results of physical techniques applied to freeze-dried materials. Mayersbach discusses at length the problems of handling freeze-dried sections prior to the application of various histochemical techniques. He draws attention to a number of possible artifacts due to post-fixation and states clearly that the proteins in freeze-dried tissues cannot be regarded as " native."

The possibilities of treating the unfixed deparaffinized sections with reagents or fixatives in the form of vapour has received some attention. Mancini (1948) studied the distribution of glycogen in freeze-dried sections by exposing them to the vapour of iodine after the removal of wax. As an alternative to the above, he used a solution of iodine in a non-polar solvent. Formalin vapour and osmic acid vapour suggest themselves as useful fixatives for freeze-dried sections when it is necessary to avoid contact of the unfixed material with any solvent, and Lacy and Blundell (1954) incorporated into their Stowell type of apparatus an additional inlet through which formaldehyde vapour could be admitted at the end of the drying process. Presumably the presence of some water vapour together with the vapour of the fixative is essential.

In studying the distribution of dyes introduced *intra vitam*, freeze-dried material has been found particularly good for the purpose. An embedding medium should be chosen which is soluble in a fluid in which the dye is insoluble, and final examination can be carried out in this same fluid. In this way Catchpole, Gersh and Pan (1950) investigated the distribution of intravitally injected Evans blue in the connective tissues of the rat. For determining the distribution of inorganic substances freeze-dried material is also excellent. Packer

and Scott (1942) used micro-incineration methods for calcium and sodium, and these entirely avoid the question of dewaxing and fixation. It is probable that cytochemical methods for inorganic ions, such as those employed by Gersh (1948), allowed too much diffusion of inorganic salts to occur during the reaction for critical localization to be achieved. Even so, the only chance of obtaining reasonably accurate localization is by the use of freeze-dried sections.

Examination after Fixation. Once again, the choice of fixative for application to the mounted sections will be dictated by the nature of the components we wish to study and the tests which it is proposed to carry out. For purely morphological studies with conventional staining, fixation in 70–80 per cent. alcohol for 16–24 hours is usually employed, after rapid removal of wax with light petroleum. This fixative is also useful if histochemical tests for simple proteins and amino-acids are proposed. We know very little of the action of alcohol on freeze-dried proteins, or, for that matter, on native proteins, but in sections the former are not aggregated as are the proteins in undried tissues. Very little morphological difference is observed between unfixed freeze-dried cells, examined by physical means in an inert medium, and similar cells fixed in alcohol and stained histologically or histochemically (Fig. 22). From these points of view, there is therefore little objection to the use of alcohol as a fixative for freeze-dried material. The use of 100 per cent. acetone is similarly free from objection and it may be the best fixative for some enzymes which are more sensitive to alcohol.

In some cases the preservation of enzymes in freeze-dried tissues is particularly good, probably approaching 100 per cent. Doyle (1950) for instance, found that the peptidase activity of rabbit appendix, using alanylglycine as substrate, was unaffected by freeze-drying and also by subsequent embedding in paraffin. If the various histochemical methods for the localization of enzyme are applied to unfixed sections, diffusion artifact is considerable. This is because the enzyme itself diffuses into the incubating medium and produces its products there, the insoluble ones being distributed at random throughout the section. So much material is lost into the incubating fluid from unfixed sections that the general microscopic appearances are very poor. Brief fixation in alcohol or acetone (1–2 hours) is sufficient to reduce the subsequent diffusion of many enzymes without interfering with their function. Even labile enzymes are partly preserved by such treatment, and alkaline phosphatase is particularly well preserved. Acid phosphatase, on the contrary, is less well preserved by freeze-drying and short acetone fixation than by some more conventional techniques (Chapter XV, p. 438). Figs. 23 and 24 illustrate the difference, in renal localization of alkaline phosphatase, between paraffin-embedded material

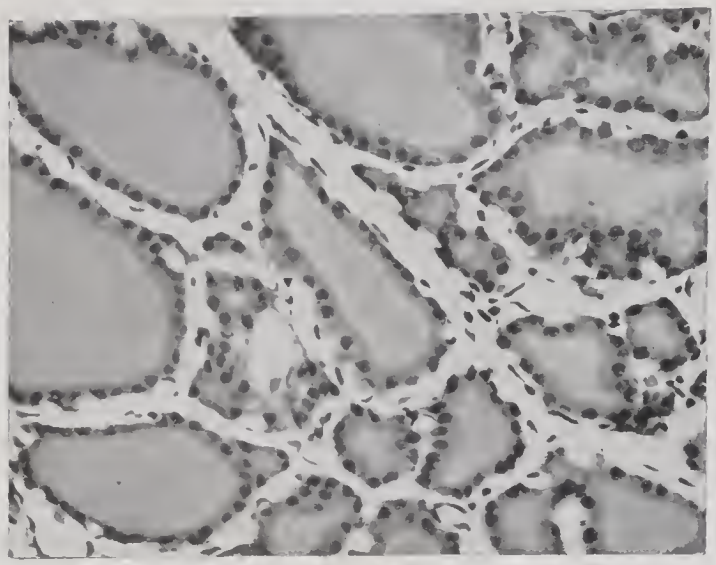

FIG. 22. Freeze-dried, alcohol-fixed, rat thyroid. The evenly stained colloid shows no shrinkage vacuoles. Hæmalum and eosin. × 200.

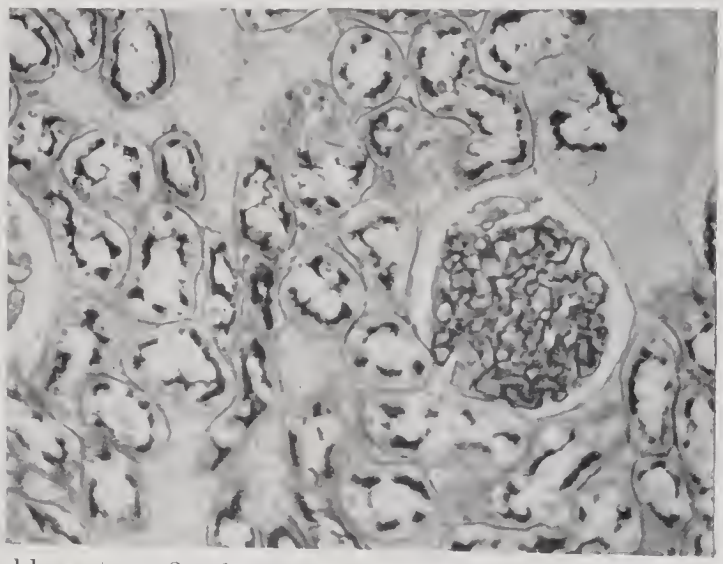

FIG. 23. Cold acetone-fixed, paraffin-embedded, rat kidney (6 μ section). Localization of alkaline phosphatase by the Gomori Ca-Co method. Incubation time 2·5 hours. × 175.

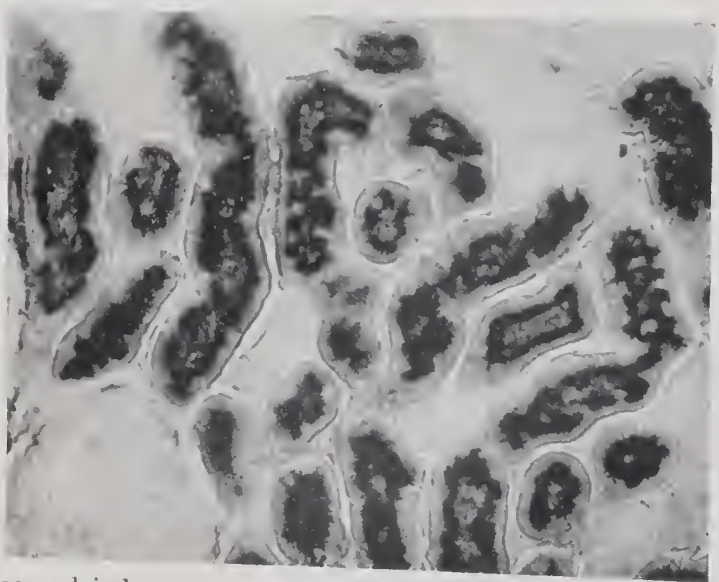

FIG. 24. Freeze-dried, acetone-fixed, rat kidney (6 μ section). Localization of alkaline phosphatase by the Gomori Ca-Co method. Incubation time 2·5 hours. × 175.

[*To face p.* 46.

conventionally fixed in cold acetone and freeze-dried material paraffin-embedded and briefly fixed in acetone before incubation. Reale (1955a and b) has compared freeze-dried with cold knife sections in a Gomori-type procedure for alkaline phosphatase. He finds the former to be superior in most respects.

The specific cholinesterases are very poorly preserved in most tissues after freeze-drying and in brain (Naidoo and Pratt, 1957) they are destroyed. Sufficient succinic dehydrogenase activity remains to give a positive result with sensitive methods (Chapter XX, p. 568), but localization of the formazan, which is the final product of the reaction, does not always correspond to that seen in fresh tissue sections.

Although theoretically freeze-drying should be an ideal method for histochemical study of enzyme systems it is becoming increasingly evident that it is not. This is because severe damage is done to many labile enzymes at various stages in the process, and not by any means all of the damage can be attributed to the final stages of embedding, mounting and dewaxing. Freezing and thawing alone are known to affect several enzymes and the denaturation effect on lipoproteins is probably very significant.

For studies on mucoproteins such as those of the anterior pituitary gland, after removal of paraffin, fixation by means of alcohol or acetone will be found inadequate. These mucoproteins remain entirely soluble in water after precipitation by alcohol or acetone and other precipitant fixatives, like picric and trichloroacetic acids, leave them in a similar state. We are therefore obliged to use formalin to obtain adequate preservation and, in practice, immersion in 10 per cent. formalin for 10–60 minutes is found to preserve mucoproteins very well. It is probable, however, as stated by Hoerr (1936) that no aqueous protein fixative acts fast enough to prevent the prior action of water vapour on the dehydrated tissue. For this reason formalin-alcohol mixtures are recommended for the study of water-soluble mucoproteins. Plate Ia shows a freeze-dried section of pancreas fixed in such a manner and subsequently stained by the periodic acid-Schiff method and toluidine blue.

Although the mucopolysaccharides as a class are readily soluble in water, tissue mucopolysaccharide-protein complexes are, on the whole, much less soluble than the mucoproteins. Catchpole (1950) found that the periodic acid-Schiff reactive material of ground substance (see Chapter IX, p. 262) was remarkably insoluble in buffers from pH 3·6–11·2, and in ammonium sulphide, dilute ammonia, sodium hydroxide, alcohol, pyridine and chloroform/methanol mixtures. It can therefore be studied in freeze-dried sections either unfixed or fixed by brief immersion in absolute alcohol. The presence of formalin is unnecessary. Epithelial mucins and premucins can also be studied in unfixed or alcohol-fixed material.

Glycogen is the only naturally occurring polysaccharide which we need to consider in animal tissues. It is well preserved by the freeze-drying technique and can be demonstrated in unfixed sections by the method of Mancini, referred to above. Other staining techniques for glycogen cannot be applied unless the material is first deparaffinized and then fixed in alcohol, and staining must be carried out with celloidin protection to prevent diffusion of glycogen out of the section. With freeze-drying, the classical streaming artifact is no longer observed (see Fig. 27, p. 64). Instead, glycogen is distributed throughout the cytoplasm in diffuse form and in granules generally smaller than those observed with ordinary fixation (see Fig. 26). The preservation of lipids has been discussed in Section 3 of this chapter, and the methods detailed there are the methods of choice. One may mention finally, however that even after dewaxing of sections by brief immersion in light petroleum, simultaneous fixing and staining in a 70 per cent. alcoholic solution of Sudan black B reveals that lipids of the phosphatide class, some presumably derived from lipoprotein complexes, are very well preserved. This method may be used qualitatively, with reservations, but it is unsuitable for comparative studies between different sections.

Examination by Autoradiography. The application of autoradiographie techniques to freeze-dried materials is considered in Chapter XXVII.

The Principles and Practice of Freeze-Substitution

In order to avoid the need for expensive apparatus for dehydrating quenched tissues Simpson (1941a and b) suggested dissolving the ice in such tissues by placing them in liquid dehydrating agents at low temperatures. He originally recommended methyl cellosolve or ethyl alcohol for the latter purpose, at temperatures between — 40° and — 78°. There are thus two essential types of freeze-substitution, one in which the quenched tissue is subjected only to dehydration and the other in which it is dehydrated in a fluid which is also a fixative. In the latter case the amount of fixation which occurs is governed by the temperature reached during the process. Absolute ethanol, for instance, may be used at — 70° without fixing the tissues at all. Ethanol/water mixtures, however, will fix to some extent even at this temperature.

The process which we are considering has been called by many different names by different authors, and even if we allow for translations three or four remain. Lison (1949) used the term *congelation-dissolution*, while Blank, McCarthy and DeLamater (1951) described the process as *non-vacuum freezing-dehydrating*. Persson (1952) preferred *freezing-dehydration in liquid medium*, and Baud (1952) *fixation "par substitution."* Gourévitch (1953), on the other hand, used the general term *congelation-dehydration* to cover freeze-substitu-

tion as well as freeze-drying, while Woods and Pollister (1955) were content with *ice-solvent drying*. Peyrot (1956) was content with a straight translation, *congelamento-sostituzione*.

Of the terms quoted above I consider that only two merit final consideration as headings to this subsection of Chapter III. These are freeze-substitution and ice solvent drying. The latter is brief, and accurately descriptive of the second half of the process. The former describes accurately the first half of the process and uses to describe the second half a single term which, though less accurate than ice solvent drying, is sufficiently comprehensive to include all variations of technique. It has, moreover, the merit of priority, and for this additional reason I have preferred it to the other.

It is not necessary to discuss here the various devices used in different laboratories for maintaining the substituent fluid at the selected temperature during dehydration. Some of the procedures, however, are given in full detail in Appendix 3, p. 786. Below, in Table 1, are set forth in abbreviated form a number of individual variations in the technique of freeze-substitution. It may appear that the differences recorded are not great. Nevertheless it is possible to produce quite extensive variations in the final picture by relatively minor alterations in technique.

TABLE 1

Techniques of Freeze-Substitution

Author(s)	Quenching	Substituents	Temperature
Simpson (1941)	Isopentane-liquid N_2	Ethanol or methyl cellosolve	− 40° to − 78°
Lison (1949)	" As for freeze-drying "	Ethanol or Gendre fluid	− 40°
Russell *et al.* (1949)	Isopentane-liquid N_2	Ethanol with metal salts	− 78°
Blank *et al.* (1951)	Propylene glycol − 20°	Propylene glycol	− 20°
Persson (1952)	Isopentane-liquid air/N_2	EtOH/MeOH 1 : 1	− 31°
Baud (1952)	Isopentane-liquid N_2	Acetic alcohol	− 40°
Gourévitch (1953)	Ethanol − 65°	Ethanol	− 20°
Woods and Pollister (1955)	Isopentane-liquid N_2	Ethanol	− 41° to − 45°
Peyrot (1956)	Acetone/CO_2	Rossmann's fluid	− 60° to − 65°
Hancox (1957)	Isopentane-liquid N_2	*n*-butanol	− 38°
Feder and Sidman (1958)	Acetone/CO_2	Osmic-acetone	− 70°

Most of the authors quoted above have reported excellent preserva- referring to plant tissues in particular, claimed that the results were

fully equal to those obtained by any chemical fixation and standard dehydration, and Blank *et al.* (1951) showed that with their method not only was cytological detail preserved but that there was no loss of water-soluble substances such as radiophosphorus. They regarded ^{32}P as water-soluble, although presumably only a small proportion of the total amount in their tissues was present in unbound form. Lison (1953), Persson (1952) and Peyrot (1956) have shown that preservation of glycogen is excellent, and I can confirm that the distribution of this substance in freeze-substituted tissues very closely resembles that seen in freeze-dried material. Peyrot (1956) has compared his results (quenching at — 80°, substitution at — 60°) with those of Lison, and he notes that with the latter's method ice-crystal artifact is sometimes considerable. With the lower temperatures used by Peyrot glycogen was always diffusely intracellular in situation and ice crystals were not visible. This author also noted that intracellular organelles, such as mitochondria, might well be present after substitution by a solvent which would destroy them at room temperature.

Few fundamental studies of the mechanism of freeze-substitution have been made and our knowledge of the physics of the process is meagre. Persson (1952) used a standard model for his tests, composed of cylindrical slices cut from a bar of 14 per cent. gelatin containing 1 per cent. dextran. The water content of his slices was determined by weighing. It was noted that the dehydration time was shortest when the model blocks were quenched at — 170°, and Persson explained this as being due to the production, at low temperatures, of a solid system of the colloids approaching a non-crystalline structure. He supposed that these were more easily dehydrated than the crystalline systems formed at higher temperatures. Bell (1952a), however, remarks that it is not known at what stage the substituent is exchanged for the water in the tissues. If, as seems likely, this takes place during the warming-up phase then diffusion artifacts are possible. Studies on the fundamental effects of freeze substitution carried out by Haneox (1957) on various tissues from the rat suggested that protein denaturation was practically absent from tissues quenched at — 160° and subsequently dehydrated in *n*-butanol for 3 days at —38°.

I believe that as far as mammalian tissues are concerned the chief indication for freeze-substitution is the preservation *in situ* of high M.W. polysaccharides, either natural, like glycogen, or artificially introduced, like dextran (see Chapter XXV, p. 653). Although the process has been used successfully for the retention of water-soluble isotopes, I confess I should feel happier in this case to use freeze-dried material. For morphological studies on both animal and plant tissues freeze-substitution is an excellent method, though it works better, as Persson has observed, with some tissues than with others. Many

authors have testified to the preservation of mitochondria, plastids and other fine intracellular structures in almost the form seen in living tissues.

Technical details of some of the methods mentioned above will be found in Appendix 3, p. 785.

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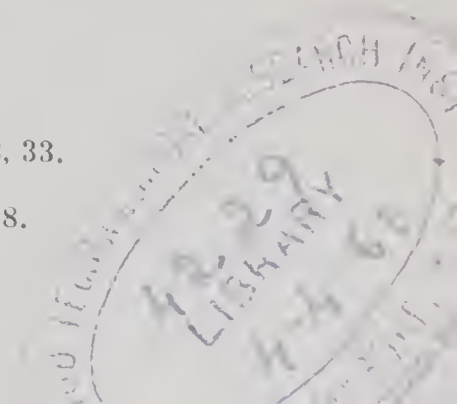

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CHAPTER IV

THE CHEMISTRY OF FIXATION

The morphological effects of the various fixatives are not considered here, since they are adequately dealt with in most general works on histological methods and particularly thoroughly by Professor J. R. Baker in his book on cytological technique (3rd Edition, Oxford, 1950). This chapter will be divided into two sections. In the first, the chemical aspect of fixation, by formaldehyde and other fixatives, is considered inasmuch as it affects the performance and interpretation of histochemical reactions. In the second, the choice of fixative for a variety of modern histochemical techniques is reviewed.

The best method of fixation for each particular method is of paramount importance and, if the standard fixatives continue to be applied as a preliminary to histochemical studies, it is essential to know as precisely as possible their effect on the reactive groups of the various tissue components. The most important of these components are the proteins, and since the best protein fixative is formalin, the actions of formalin are considered most fully in the section which follows. Only where strong objections to the use of formalin can be raised will there be any necessity to replace it by fixatives of other types. For histochemical purposes alcohol and acetone are the only remaining fixatives of much importance, metallic salts are less often used though they are occasionally essential. Fixation with osmium tetroxide mixtures will be considered in this chapter, where it properly belongs, rather than in Chapter XXIX, where electron histochemistry is discussed.

The Chemical Actions of Fixatives on Tissue Components

Formalin

The reactions of formalin with tissue proteins are numerous and complex, since it can combine with a number of different functional groups, in many cases forming bridging links between them (see Table 2, below). To this function of forming links between adjacent protein chains formalin owes its success as a polymerizing fixative. According to French and Edsall (1945) the most frequently encountered reaction of formalin is its addition to a compound containing a reactive hydrogen atom with the formation of a hydroxymethyl compound.

$$RH + CH_2O \rightleftharpoons R.CH_2(OH)$$

The compound is usually reactive also and it may condense with a

further H atom to form a methylene bridge (—CH_2—) in the manner illustrated below.

$$R.CH_2(OH) + HR' \rightleftharpoons R{-}CH_2{-}R' + H_2O$$

These methylene bridges, as the above reaction suggests, are readily ruptured by hydrolysis. Many of the combinations of formalin with tissue proteins are reversible by the simple process of washing or, for instance, by the application of dimedone (see Chapter XII, p. 342). Others are irreversible.

The groups particularly involved in the fixation of proteins by formalin are amino, imino and amido, peptide, guanidyl, hydroxyl, carboxyl, SH and aromatic rings. Methylene bridges may be formed between two similar groups, say NH_2, or between NH_2 and peptide (CONH), or between NH_2 and NH, to quote a few examples. Their occurrence is dependent on the presence of a second suitable group with a suitable spatial relationship to the formaldehyde addition compound formed by the first. Interesting details are available of the action of formalin on some of the commoner proteins. Between pH 6 and 8 formalin reacts with keratin without affecting the S—S links of cystine. In more alkaline solutions, however, it is considered to reduce S—S to two SH groups and subsequently to react with these forming, in some cases, a methylene bridge (S—CH_2—S) in place of the original disulphide link (Middlebrook and Phillips, 1942 a, b). From experiments in the tanning of casein by formalin at pH 6 Nitschmann and Hadorn (1943, 1944) deduced that a condensation between the ϵ-amino groups of lysine and the peptide links of another chain was responsible for most of the observed combination with formalin. (Leather chemists, it may be noted, tend to speak of the fixation of formalin by proteins, a reversal of the usual histological terminology.) These same authors (1943) showed that a considerable amount of the formalin remaining bound to protein after 5 hours washing in running water could be removed by further washing (up to 24 days). Even after a period such as this (which is obviously well beyond the upper limit of washing in histological and histochemical practice) some formalin remained in irreversible combination with protein. This was freed by prolonged hot acid hydrolysis and collected for estimation by distillation. A sample of casein washed for 12 days was shown to contain 1·9 per cent. of formalin and a similar sample washed for only $1\frac{1}{2}$ hours contained 2·6 per cent. Obviously something approaching the latter percentage would remain bound to protein in most histological preparations in the block stage at the point of dehydration in alcohol. Neither the amount removed by the further processes of embedding, nor the amount of washing required before the percentage of formalin in the mounted section approaches the lower of the two figures quoted above for

bound formalin content, has been determined. Probably very little additional washing of the section is required.

The significance of these observations in histochemical practice is that treatment of proteins with formalin, followed by washing, is likely to leave the majority of active groups in a condition to react with any reagents we may have occasion to use. The Sakaguchi reaction for arginine (Chapter V, p. 119), for instance, is considerably modified in the presence of formalin, which appears to alter the guanidyl group in such a way as to block the action of hypochlorite upon it. Histochemically, however, the reaction can be performed on formalin-fixed sections, from which the excess formalin has been removed by washing, in a perfectly satisfactory manner. Even if we propose to apply spectrophotometric measurement to make the reaction quantitative, and even if we are uncertain whether a proportion of the guanidyl groups is still blocked in the washed formalin-fixed sections, the application of a standard technique of fixation will allow what will usually be a valid comparison between one tissue and another.

Most of our knowledge of the action of formalin on tissue compounds comes from studies made in connection with the tanning and wool industries, so that collagen and keratin are the two substances we know most about. A great deal of this work has been done with formalin under quite unhistological conditions (e.g. 100° at pH 1, 70° at pH 4, various temperatures at pH 10) and Middlebrook (1949) showed that the amount of formalin irreversibly bound by various proteins dropped sharply, in most cases, when the pH rose above 1·0. This was true, for example, of casein, gelatin and wool but in the case of carboxyhæmoglobin the amount of bound formalin rose to a maximum at pH 4. Middlebrook connected this with the formation of bridges involving the amide groups of asparagine, present in large amounts in hæmoglobin. In histological and histochemical work formalin is nearly always used in buffered solutions at or above the neutral point, and Wolman (1955) has suggested that part of the effectiveness of these higher levels is due to the rapid conversion, in neutral and alkaline solution, of the polymerized form of HCHO to the monomer. In water the latter exists in hydrated form as methylene glycol ($CH_2(OH)_2$), and this is considered to be the more effective fixative of the two. The reactions of formalin are set forth below in Table 2.

This list does not exhaust the possible reactions of formalin with groups present in amino-acids and a number of other reactions have been described using pure amino-acids. Such reactions probably do not take place in materials of the type we are dealing with, but the reactions indicated in the Table certainly do occur. It will be observed that the only irreversible reaction is that which takes place with the aromatic hydrogen in tryptophan, tyrosine, phenylalanine and histi-

TABLE 2

Reactions of Formalin

(mainly after French and Edsall, 1945)

Group	Reaction	Remarks
Active hydrogen	$R.H + CH_2O \rightleftharpoons R.CH_2OH$	—
Active hydrogen + condensation	$R.CH_2OH + H.R' \rightleftharpoons R—CH_2—R' + H_2O$	Methylene bridge
Amino (1 mol)	$R.NH_2 + CH_2O \rightleftharpoons R.N=CH_2 + H_2O$	—
Amino (2 mols)	$R.NH_2 + 2CH_2O \rightleftharpoons R.N(CH_2OH)_2$	—
Imino	$R_2=NH + CH_2O \rightleftharpoons R_2=N.CH_2OH$	—
Amide	$R.CONH_2 + CH_2O \rightleftharpoons R.CO.NH.CH_2OH$	—
Peptide	$—CO—NH + CH_2O \rightleftharpoons CO.N(CH_2OH)$	—
Guanidino	Not known	Probably a bridge with NH_2
Hydroxyl (a)	$R—OH + CH_2O \rightleftharpoons R.O.CH_2OH$	Hemiacetal formation
Hydroxyl (b)	$2R—OH + CH_2O \rightleftharpoons RO.CH_2.OR$	Acetal formation
Carboxyl	$2R—COOH + CH_2O \rightleftharpoons (R—COO)_2CH_2 + H_2O$	Unimportant in aqueous solution
SH (a)	$R.SH + CH_2O \rightleftharpoons R.S.CH_2OH$	Semithioacetal
SH (b)	$2RSH + CH_2O \rightleftharpoons (R.S)_2CH_2 + H_2O$	Methylene bridge (thioacetal)
Aromatic H	$\diagdown CH + CH_2O \rightarrow C\diagdown — CH_2OH$ $\diagup \qquad \diagup$	Stable C—C bond

dine. This reaction occurs very slowly and is often followed by condensation with another reactive group to form a methylene bridge. The phenol formaldehyde polymers are formed in this manner (see Chapter XXIII, p. 649). It is worthy of note that when formalin is used as a histological fixative, the process of fixation, as judged by purely histological standards, is not complete until at least 7 days have elapsed.

It is clear that in using neutral 10 per cent. formalin at room temperature, and even more so at 4°, we are making relatively little use of its capacity to form addition compounds and bridges. Moreover, a high proportion of those initially formed are too labile to withstand washing. Unfortunately we do not know precisely which groups are responsible for the irreversibly-bound formalin fraction of washed tissues, a matter of importance since it is these groups whose reactivity is suppressed. It is likely, however, that aromatic hydrogen and NH_2 groups, by bridge formation, are the two which are mainly involved.

Metallic Ions and Complexes

Chromium. There is some modern evidence of the action of the protein-precipitant metals which is worth considering. In histology and histochemistry we are mainly concerned with osmium, mercury and chromium and once again, due to the volume of investigations

carried out by the tanning industry, much more is known about the mechanism of fixation with the last of these. Chromium salts have the property of forming complexes with water of the type Cr—O—Cr, and these complexes combine with the reactive groups of adjacent protein chains to bring about a binding effect similar to that of formalin (see Gustavson, 1949, 1956). The main affinity of chromium is for the carboxyl and hydroxyl groups of protein and Bowes and Kenten (1949) found that no chromium is fixed by methylated collagen from solutions of chromium sulphate at pH 4·0. (The effect of methylation is to block carboxyl groups by esterifying them.) Similarly, Gustavson (1940) showed that only small amounts of chromium were taken up by collagen at pH 1 ; at this pH the carboxyl groups no longer maintain a negative charge and they therefore fail to react. The importance of carboxyl is stressed also by the work of Strakhov (1951), who showed that collagen can fix much more chromium than silk fibroin (which contains fewer COOH and NH_2 groups) and that an ϵ-caprolactam polymer containing neither of these groups fixed no chromium at all. Green (1953) considers that the primary reaction between the chromium complex and carboxyl groups is followed by the formation of co-ordinates with OH and NH_2 groups. Hexavalent chromium has been shown by Grogan and Oppenheimer (1955) to be bound by egg albumin in decreasing amount as the pH is changed from 4 to 7·35. Gustavson (1949) considered this effect to be due to the larger size of the complexes at acid pH levels and to the increased availability of ionized carboxyl groups.

No histological fixatives containing chromium have a pH lower than 2·9, and the effect of chromium salts in neutral or moderately acid solution will be to form complexes with tissue proteins of the type described. The final effect of these is to break up the internal (salt) links of the protein and to increase the number of basic groups available in a reactive state, thus increasing the acidophilia of the whole structure. Since we have no exact measure of the effect of chromium on the various reactive groups, chromium-containing fixatives, with some specific exceptions, are best avoided in histochemical studies. These exceptions are the use of chromium trioxide as an oxidizing agent in the production of the so-called chromaffin reaction (Chapter XXIII, p. 637), its possible use for the preservation of glycogen, and the use of $K_2Cr_2O_7$ for lipids and nucleic acids in the process of controlled chromation (see p. 64, and Appendix 4, p. 790).

Mercury. Salts of mercury are much more commonly employed in histological fixatives and in many institutions (my own, for instance) the routine fixative is a mercurial one. Histochemical studies may be called for when no material other than that fixed with mercury (formol-mercury) is available. It is therefore important to consider the effect

of salts of this metal on the tissues, and particularly on the proteins of these. In general, Hg^{++} behaves like other metallic ions in combining with the acid groups of proteins, especially carboxyl and hydroxyl, and the phosphoric acid of nucleoproteins. It differs from chromium in not forming complexes capable of binding together adjacent protein chains and also from the majority of metals in one important respect. This is in its selective affinity for thiol (SH) groups. If a very small quantity of a mercuric salt is added to a protein containing reactive thiol groups it will react with these in preference to any others, and the stability of the mercury-sulphur bond is greater than that between mercury and any other grouping. Hughes (1947) prepared a fraction of serum albumin which contained one SH group per molecule. When this was allowed to react with a mercury salt the resulting protein mercaptide was found to contain $\frac{1}{2}$ an atom of mercury per albumin molecule, suggesting that the unit formed was an albumin dimer. The reactions which take place (modified from Hughes, 1950) are given below.

$$RSH + HgCl_2 \rightleftharpoons RSHgCl + HCl \quad \text{and}$$
$$RSHgCl + RSH \rightleftharpoons (RS)_2Hg + HCl$$

The second reaction is a slow one and probably of little importance in histological fixation. Both reactions are reversible, but while the second can be reversed by any reaction which forms an undissociated mercury complex, the first is only reversed by reagents such as cysteine which form equally stable mercury derivatives.

These findings are not immediately applicable to the problem of mercury-fixed proteins in histochemistry, since in ordinary proteins, in contradistinction to Hughes' serum albumin fraction, not all the SH groups present are available for reaction. They do suggest, however, that complete reversal of mercury-binding of tissue groups might be affected by means of reagents like cysteine. For histochemical purposes the important point is whether the reaction of mercury with the various protein groups is reversible by the ordinary processes of embedding, washing, and removal of coarse mercury precipitates by means of iodine and thiosulphate. While specific investigations into this point have not been made, it is certain that some mercury remains bound by the acid groups in nucleoproteins and other proteins and it is probable that some remains bound to SH groups also. It is, therefore, advisable to avoid the use of mercurial fixatives in critical histochemical practice if investigation into the nucleic acids or sulphydryl groups is proposed. Other protein reactions (e.g. Millon, Sakaguchi and tetrazonium) can be performed in spite of the presence of mercury.

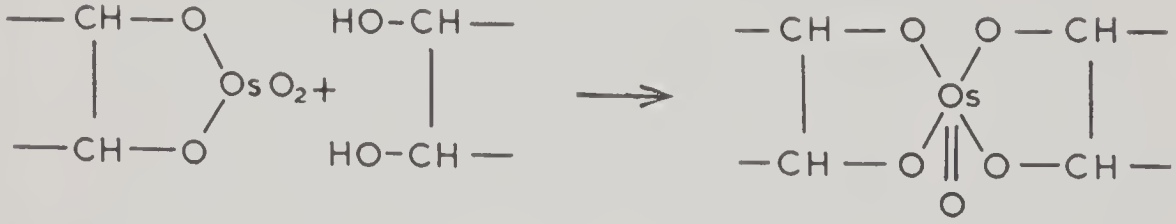
Osmium. The so-called osmic acid was employed by Schultze and Rudneff in 1865, and since that time osmium tetroxide (OsO_4) has been

in general use as a cytological fixative. Its use in histology has been limited by its poor penetrating qualities, but its current pre-eminence in the preparation of tissues for electron microscopy has brought about a revival of interest in the mechanism by which it acts as a fixative. Our knowledge of this process is still scanty and unsatisfactory in many respects.

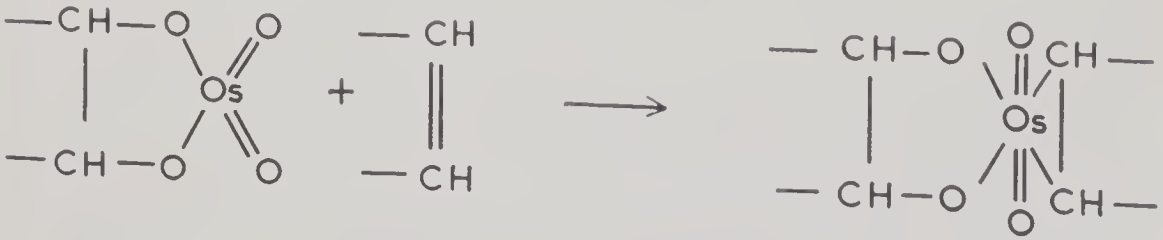
Unsaturated fats reduce OsO_4 with the formation of black compounds containing osmium or its hydroxide, and Criegee (1936) has suggested that this is due to oxidation of the double bonds between adjacent carbon atoms.

(colourless) (black)

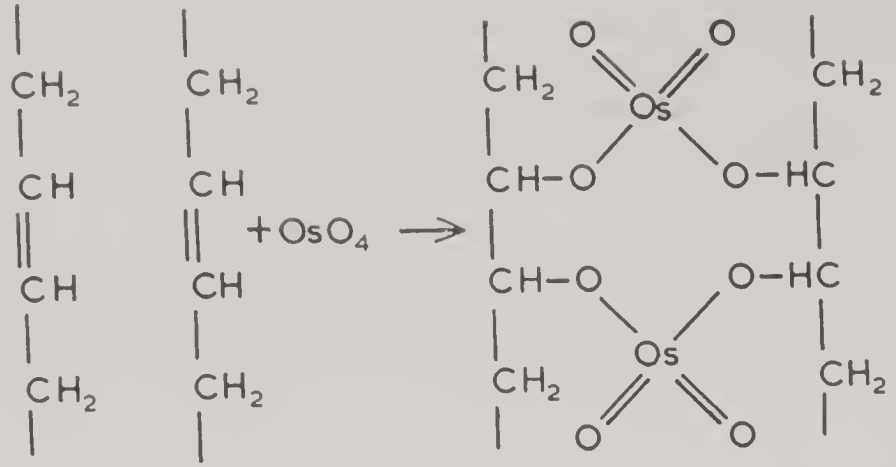
This results in the formation of a bridge (monoester), and it has been stated that similar bridge formation can take place between adjacent aliphatic hydroxyls (1 : 2-glycols) after oxidation. The other end of the molecule shown in the equation above is free to react if an oxidized ethylenic group (diol form) is available, with the formation of a diester.



An alternative hypothesis has been put forward by Wigglesworth (1957), who considers that in place of the second reaction the OsO_2 may form a double co-ordinate linkage directly with the carbon of the double bond.



If two bonds in adjacent chains are in close apposition Wigglesworth considers that double monoester formation may occur, as shown below, with consequent binding of the fatty acid chains.



Whatever the nature of the reaction of unsaturated fats with OsO_4 polymerization will occur if, as in triolein, there are a number of unsaturated chains in the molecule. It may also occur when, as in linoleic acid, there are a number of unsaturated bonds in a single chain.

Porter and Kallman (1953) repeated the studies of Fischer, Mann, Baker and others and showed that 2 per cent. OsO_4 formed gels with albumin, globulin and fibrinogen. The clear gel which forms relatively slowly with albumen they regard as an indication of fine micellar or even unimolecular binding. The opaque gel formed with globulin and fibrinogen, on the contrary, is held to indicate some coarser type of binding. Wolman (1955) believes that the bridging which occurs in the early stages of fixation with OsO_4 is responsible for the excellent preservation of structure, and that with longer fixation damage occurs through over-oxidation. He also stresses the fact that the well-known reversal of tissue acidophilia must be due to blockage or destruction of NH_2 groups. Finean (1954) used buffered OsO_4 at pH 7·0 to fix sciatic nerve and observed an increase in intensity and a change in relative intensity of the bands in its low angle X-ray diffraction pattern. He suggested that the monoethanoid fatty acids of the myelin sheath were the main site of its action. Alcohol normally extracts the lipids of the myelin sheaths completely, according to Finean. After OsO_4 fixation it no longer does this and it is assumed that binding of lipid to protein has occurred. This would be unnecessary, however, if the osmium-lipid compound were insoluble in alcohol.

In a study of the reactions of OsO_4 (and RuO_4) with biologically important substances Bahr (1955) tested hundreds of compounds *in vitro* with OsO_4 at a final concentration of 0·003 M, in water or carbon tetrachloride at 0°, 20° and 50°. The substances tested had a final concentration of 0·6 M. The main positive reactions were with cysteine, triethanolamine, oleic acid, methionine, arginine, ornithine, lysine and ascorbic acid. Bahr comments on his results : (1) *Saturated hydrocarbons* react slowly or not at all ; (2) *halogen derivatives* do not react ;

(3) *alcohols and ethers* react rapidly. With hydroxyls the effect is maximal with 3 to 5 carbon chains, while with unsaturated alcohols it increases with the number of double bonds but decreases as chain length goes up ; (4) *SH and SS* react immediately ; (5) *amines* react increasingly as chain length increases ; (6) *aldehydes and ketones* only react when present in long chains. The approximate order of reactivity was found to be SH>C = C> terminal NH_2>SS, CHO, terminal OH> adjacent aromatic OH. Nucleic acids and carbohydrates were inert. Further work by Wolman (1957), however, has shown that under certain conditions OsO_4 is reduced by amino-sugars. This author tested a number of pure compounds *in vitro* with aqueous OsO_4, with an OsO_4/oxidant mixture (Swank and Davenport, 1935), and with OsO_4 in non-polar solvents. He observed three types of reaction : (1) the formation of a black precipitate with watery solutions of OsO_4 (unsaturated lipids, SH groups, etc.) ; (2) the formation of a black precipitate with OsO_4 in a non-polar medium only (NH_2 compounds in certain forms) ; (3) oxidation by OsO_4 without the formation of a visible precipitate (polysaccharides containing available 1 : 2-glycol groups).

The above findings show that the absence of a black colour cannot be taken to indicate the absence of a reaction and they throw some light on the mechanism of Marchi staining (discussed in Chapter XI, p. 309). We are left, however, without any satisfactory explanation for the efficiency of OsO_4 as a fixative. Dallam (1957) has investigated the problem of protein loss from tissue components during the processes of OsO_4 fixation, dehydration, and embedding in methacrylate. In the case of a sample of rat liver mitochondria, for instance, he found a 22·6 per cent. loss of protein during fixation and a further 12·2 per cent. during dehydration. The loss of phospholipid in the fixative could not be calculated for technical reasons, but in the subsequent dehydration stage it was 27 per cent. Not only do these findings indicate the need for caution in interpreting electron micrographs, but also in accepting estimates of the efficiency of any fixative on the basis of histological appearances alone.

Alcohol and Acetone

These two protein precipitants have been used as " fixatives " especially in enzyme histochemistry because, in spite of the morphological disturbances which they create in the tissues, they leave the reactive groups of enzymes to a large extent in their original state. Unfortunately very little is known about the exact effect of alcohol and acetone on proteins. In general, however, denaturation of a protein by precipitation may change the reactivity of its groups in three ways : (1) by rendering parts of the molecule inaccessible to

various reagents ; (2) by altering the rate at which such reagents diffuse into the protein (not significant in tissue sections) ; and (3) by bringing into proximity previously separated groups in the protein molecule, thus producing new stereochemical relations. The effects of denaturation are to some extent reversible and many of the original properties of the protein are sometimes regained when the denaturing agent is removed. The mucoproteins of the anterior pituitary gland, for instance, are entirely soluble in water after precipitation by brief treatment with alcohol, and after treatment with acetone the alkaline phosphatase of various tissues is still soluble to some extent in dilute solutions of alcohol (see Chapter XIV, p. 388). Other effects are apparently irreversible, such as the partial inactivation of most enzymes. It is not known whether this is due to a chemical effect on their reactive groups or to one of the denaturation factors mentioned above.

The cross linkages which bind protein chains together are of several types, some of these are illustrated in Fig. 25 below.

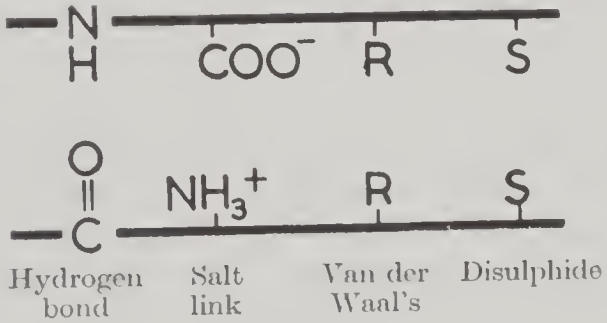


Fig. 25.

It has been suggested that alcohol acts by turning the protein chains outwards (away from each other), breaking the hydrogen bonds and salt links and thus revealing the end groups of the side chains to a varying extent. The precise mechanism, and the extent of liberation of side chains is quite unknown, however.

Although on *a priori* grounds one would imagine that alteration of the reactivity of various groups in the tissues would be greater in the case of the formalin and metal-containing fixatives than with alcohol or acetone, in practice the effect of formalin is not found to be any greater in this respect. Examples of this will be found in the following section on the use of formalin-fixed material for enzyme studies.

The Choice of Fixative

In this section it is proposed to discuss the appropriate fixation for tissues required for the various histochemical procedures listed below. No really new methods of fixation have been evolved in recent years, but advances have been made in our knowledge of the ways in which the classical fixatives work and in their application to various

problems. In particular, considerable advances have been made in the fixation of glycogen, lipids, nucleoproteins and enzymes. Mucoproteins and mucopolysaccharides will also be dealt with and the choice of fixative to precede treatment with various enzymes, when these are used as histochemical reagents.

Glycogen

It is now known that a large variety of glycogens occur naturally which are distinguished by varying degrees of polymerization. The larger molecules of the more highly polymerized glycogens are those we have been accustomed to see in tissues fixed by a wide variety of methods as well as the more or less classical alcohol-containing ones. The less highly polymerized glycogens have probably not been successfully fixed in the tissues by any of the processes employed but have diffused into the fixing fluid. Moreover, large amounts of glycogen, even after successful fixation, are lost during subsequent staining. This loss, of the type which can be minimized by using collodionized slides (see Chapter IX, p. 270), occurs first in the less highly polymerized forms, though it may ultimately involve all the glycogen in the section.

Deane, Nesbett and Hastings (1946) suggested an improvement in the fixation of glycogen which involved the use of ice-cold picro-alcohol-formalin. Lison and Vokaer (1949) recommended for the same purpose either cold alcohol or a mixture of 96 per cent. alcohol saturated with picric acid (85 parts), 40 per cent. formalin (10 parts), and acetic acid (5 parts). These fixatives were used at a temperature of — 73°, produced by an acetone-solid CO_2 mixture, and Lison claimed that they could effectively prevent polarization, that is to say, streaming of the granules to one pole of the cell. Polarization is certainly reduced to a minimum by such methods. It can otherwise only be avoided by the use of the freeze-drying technique (see Figs. 26 and 27). Alcoholic fixatives are the worst offenders in producing polarization at ordinary temperatures and also in showing a pronounced difference in the amounts of glycogen revealed in sections taken from the surface and from the depth of the block. If acetic acid is used in the fixative the granules are coarser, and with aqueous fixatives, in general, polarization and surface-depth differences are far less than when alcohol is used. Mercuric chloride decreases the amount of glycogen demonstrable by any of the methods discussed in Chapter IX. Although it has been recognized for some time that glycogen appears in formalin-fixed sections, Vallance-Owen (1948) has done valuable service in pointing out that it is quite as adequately fixed by formol-saline as by alcohol or picric-alcohol mixtures. His work was done at room temperatures (23°–25° in summer), however, so that some of the less highly polymerized forms of glycogen, which are preserved by Lison's refinement,

were presumably lost in the fixing fluid. The experiments of Lillie (1947) on the preservation of glycogen by various fixatives gave results which do not entirely agree with those of Vallance-Owen. Lillie found that rat livers fixed in neutral formalin or in acetic acid-formalin with and without mercuric chloride, contained little or no glycogen. In the cat liver, however, neutral aqueous formalin did preserve glycogen, and the author noted that unmodified absolute alcohol fixation appeared inferior to aqueous formalin fixation with respect to preservation of glycogen as well as in other regards. Neutral 10 per cent. formalin-alcohol was found to be the best of all the fixatives which he tried. Both Lillie and Vallance-Owen showed that fixed blocks of tissue could be kept in running water for 24 hours without loss of glycogen.

Lipids

Fixation of lipids other than neutral fats was greatly improved by the use of Baker's (1944) fixative, which was designed for the preservation of phospholipids. In his original method this author used formalin together with calcium and cadmium chlorides, and McManus (1946) substituted cobalt nitrate for the more expensive cadmium salt. Berg (1951), in a survey of the lipid-dissolving effects of various solvents on fixed sections, could not find any difference between routine formalin-fixed material and that fixed in formalin with the addition of calcium chloride. This observation confirms Baker's ideas of the mechanism by which calcium and other salts preserve phospholipids. Although the latter are preserved, that is to say prevented from diffusing into the fixing fluid, by Ca, Co and Cd, they are not fixed in the usual sense of the word and are still removable by fat solvents. The action of Ca, Co and Cd ions is presumed to be due to their influence on the formation of complex coacervates of phospholipid with other cellular constituents (protein, mucopolysaccharide, etc.). Coacervates (Latin : *acervus*, a heap or swarm) of the complex type can be regarded as structures resulting from the aggregation of molecules which are held together by intermolecular forces, in the form of a mosaic or lattice of lipid and protein molecules.

Two contributions to the better preservation of phospholipids have been introduced by Elftman (1954, 1957). The first, a process which its author terms *controlled chromation*, is a further refinement in a long line of methods derived from the original method of Weigert (1884) by the work of Smith and Mair (1908), Ciaccio (1909), Dietrich (1910) and Baker (1946). It depends on the fact that oxidized phospholipids are less soluble in fat solvents than their unoxidized precursors.

Four variables are subjected to control, pH being added to the usual triad of time, temperature and concentration of the reagent. The standard procedure involves fixation in 2·5 per cent. $K_2Cr_2O_7$ at pH 3·5

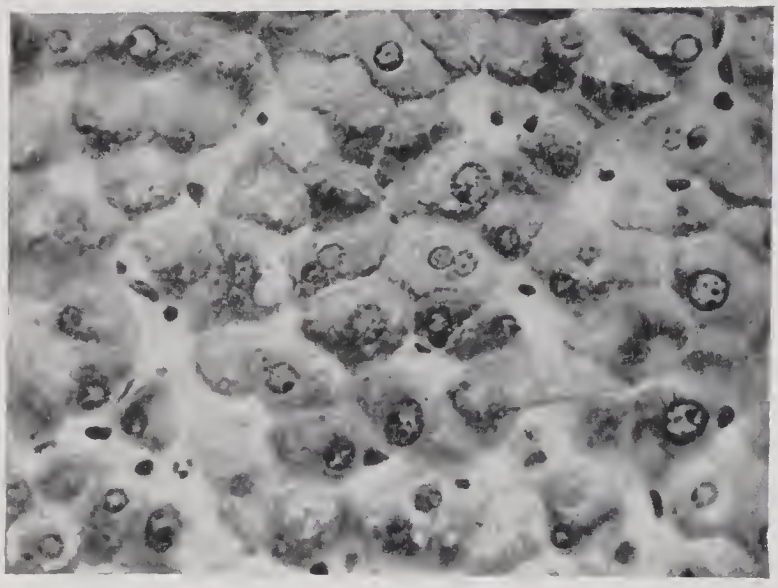


FIG. 26. Alcohol-fixed human liver (biopsy specimen). Showing polarization of glycogen due to streaming artifact. Best's carmine, hæmalum. × 350.

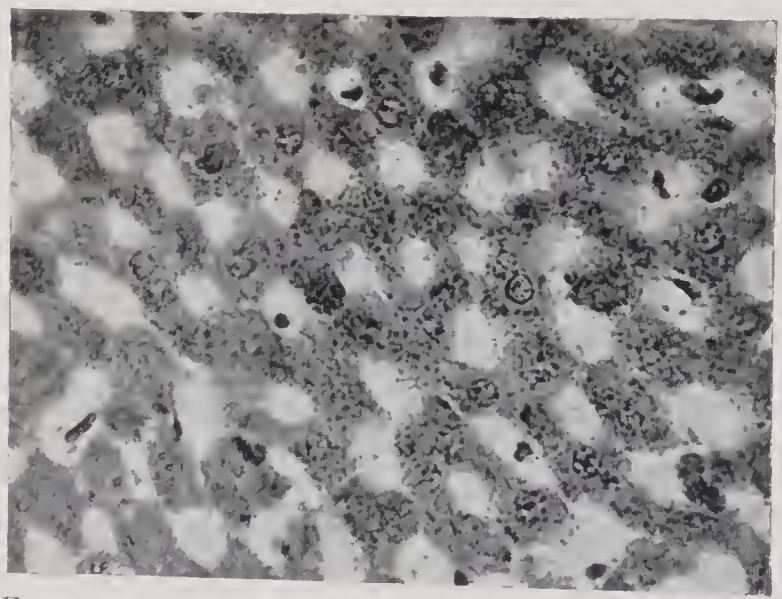


FIG. 27. Freeze-dried, alcohol-fixed, rabbit liver. The granules of glycogen are distributed throughout the cytoplasm of the cells. Best's carmine, hæmalum. × 350.

[*To face p.* 64.

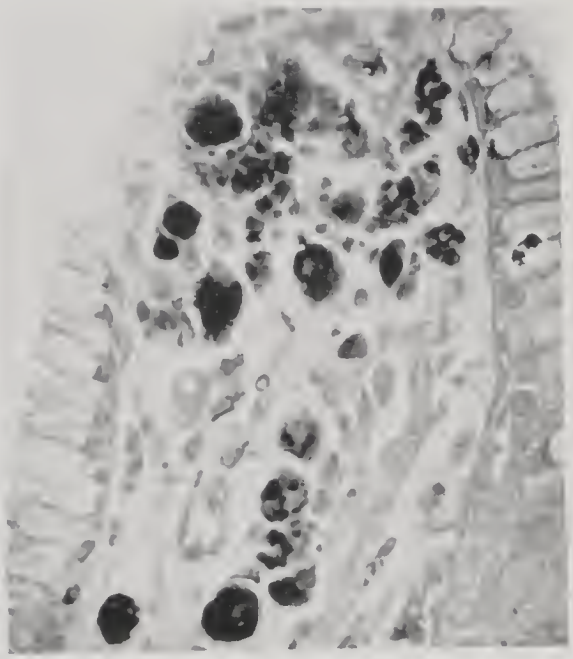


FIG. 28. Human cervix uteri. Intra- and extracellular Russell bodies. Millon reaction. × 400.

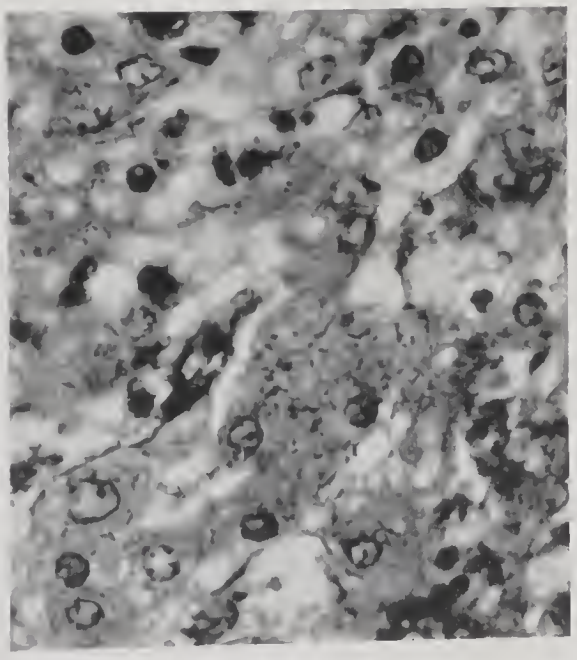


FIG. 29. Human liver, freeze-dried and alcohol-fixed. Showing result of the terephthalaldehyde-leucofuchsin method of Danielli. × 520.

(acetate buffer) and 56° for 18 hours. Paraffin sections are stained with Sudan black B and with hæmatoxylin (see Appendices 4, p. 790, and 11, p. 849). Chromated lipids are stained by both dyes, non-lipid chromated substances only by hæmatoxylin. This procedure is discussed more fully in the chapter on lipid methods (p. 313).

Elftman's second fixative (dichromate-sublimate) contains 2·5 per cent. $K_2Cr_2O_7$ in 5 per cent. $HgCl_2$. It thus closely resembles Zenker (minus acetic acid) and Helly (minus formalin) and has an initial pH slightly above 3·0. Long fixation (3 days) and paraffin embedding is followed by staining of the sections with Sudan Black B in ethylene or propylene glycol (Appendix 11, p. 855) and mounting in Apathy's medium (Appendix 1, p. 776). Dichromate-sublimate gives good results in mammalian tissues, the mitochondria are well preserved and the Golgi region shows up in many tissues as a circumscribed area of perinuclear staining.

A method evolved by Rinehart and Abu'l-Haj (1951) may be mentioned here, although it does not directly concern fixation, because it has to do with the preservation of lipids and lipid coacervates. These authors used one of the polyethylene glycols or carbowaxes which are long chain polymers of the type $HOCH_2 (CH_2OCH_2) CH_2OH$, for the dehydration and embedding of tissues after formalin fixation. Lipids, cholesterol and cholesterol esters are insoluble in solutions of carbowaxes in water, and in the carbowaxes themselves. The method gives excellent preservation of lipids and is given in full in Appendix 1, p. 773.

Nucleoproteins and Nucleic Acids

Nucleic acids, like the glycogens, exist in many different states of polymerization (see Chapter VIII) and it is certain that any method of fixation induces changes in their physical state. Changes in chemical reactivity are also brought about by the majority of fixatives so that exception can be taken to every method on one or both of these points. From the histochemical point of view formalin is not a good fixative for nucleic acids and nucleoproteins. It blocks a large number of reactive groups so that stainability by both basic and acid dyes is considerably reduced. Improvement occurs when mercury or chromium salts are added but objection must be made to these if histochemical techniques are to be employed. Both salts interfere to some extent with the action of enzymes and lanthanum acetate (Hammarsten *et al.*, 1935 ; Opie and Lavin, 1946) acts in a similar manner, preventing the action of ribonuclease altogether. Precipitant fixatives, like alcohol and acetic acid, are much more often recommended for the preservation of nucleic acids and Carnoy is the most popular of these. Little exception can be taken to it from the chemical point of view, but

White and Elmes (1952a and b) have shown that ethyl alcohol rapidly causes irreversible changes in the structure of isolated deoxyribose nucleic acid (DNA) as measured by the ability of the nucleic acid fibres to induce dichroism when stained with a variety of dyes and examined by polarized light. They found methyl alcohol to be much less active in this respect, probably because it is a weaker dehydrating agent than ethyl alcohol. The acid fixatives produce their excellent morphological pictures by precipitating the nucleoproteins and, at the same time, they progressively break the bonds between the nucleic acids and the proteins, increasing the number of acid groups available for reaction and hence the degree of basophilia. From the outset, therefore, the physical state of the nucleoproteins is profoundly altered and a long sojourn in acid fixatives causes extraction first of ribonucleic acid (RNA) and then of DNA. For this reason, prolonged fixation in Carnoy must be avoided.

Differences in fixation have long been known to necessitate different times of exposure to acid hydrolysis in performing the Feulgen reaction (Chapter VIII, p. 193), yet it is perfectly permissible to use this technique as a qualitative reaction after almost any fixative. Quantitative estimation of DNA stained by the Feulgen reaction has been carried out by a number of authors and, although the accuracy of some of the earlier results seemed doubtful, direct comparison between biochemical and spectrophotometric results gives good grounds for confidence. If comparative studies are required it is necessary to accept the fact that all fixatives cause changes in nucleic acids, and to select a fixative which will preserve, or at least modify to a constant and not too large degree, the property it is desired to investigate. The time of application should be the minimum necessary to achieve this object so that the physical and chemical characteristics of the nucleic acids will be altered as little as possible. For most purposes Carnoy will be found quite adequate ; it allows the performance of the majority of histochemical tests for proteins and amino-acids and does not interfere either with the action of enzymes or with the various salts and acids used for depolymerization. A word of warning about the effect of heat may be appropriate. Short exposure to 70°–90° (the average temperature employed during the drying of sections on slides) causes profound changes in the nucleic acids. Such temperatures must either be avoided, or else standardized, in any comparative histochemical studies.

Enzymes

The effect of fixation on enzymes continues to be of increasing importance as histochemical techniques for demonstrating them increase in number. Theoretically, the best way of showing enzyme

activity by histochemical means would appear to be by the use of fresh frozen sections or tissue blocks. There are considerable objections to both of these in practice, however, especially to the latter. With the cold knife methods (Chapter II, p. 13) it is technically difficult to obtain good fresh frozen sections of the majority of tissues, but even if these are obtained with greater ease by means of cold microtomes, there remains a further objection. Particularly if incubation with the substrate is prolonged, I have observed that loss of enzyme and of other protein and non-protein materials into the incubating medium is much greater than in fixed material. This leads not only to false localization of enzyme (due to diffusion in the section) but also to widespread deposition on the section of the product of enzymic activity in the medium, and to general filthiness of the section due to partial dissolution of its components.

During the past 15 years the histochemistry of the phosphatases and esterases has largely been carried out with materials fixed in alcohol or acetone, usually at cold-room temperatures (4°), since these two protein precipitants were found to produce only a small loss of activity in the case of alkaline phosphatase. Moreover, they preserve the tissues in such a way as to allow paraffin embedding. Danielli (1946) cut frozen sections of tissues fixed in absolute alcohol and found no significant loss of alkaline phosphatase activity. Up to 75 per cent. loss of activity was caused, however, by the subsequent process of paraffin embedding. Mowry (1949) studied the effect of certain technical procedures on the preservation of alkaline phosphatase for histochemical purposes. He noted no significant differences between acetone and alcohol and no improvement was recorded on buffering these fixatives. Their use in the cold increased the amount of enzyme preserved, and when tissues were fixed in alcohol saturated with sodium α-glycerophosphate preservation of enzyme was improved. Mowry concluded that considerable latitude was possible in the fixation of tissues for alkaline phosphatase studies, but that, on the other hand, the process of embedding did not allow such latitude. The best fixation of enzyme was achieved with acetone or alcohol-fixed blocks cleared in light petroleum (petroleum ether) and embedded *in vacuo* in ordinary or low melting-point (42°–44° C.) paraffin. He recommended that embedding should not occupy longer than 30 minutes. The relative importance of the embedding procedure has been stressed more recently by Dalgaard (1956). This author made model experiments with pieces of gelatine containing serum from dogs with obstructive jaundice. He found that the amount of phenyl-phosphatase was unaffected by fixation in 80 per cent. ethanol or by dehydration and clearing in methyl benzoate-colloidin (8 hours). The temperature of embedding, however, was critical. At 53° a sharp loss

of enzyme occurred and Dalgaard therefore advises embedding in a mixture of soft (42°) and ordinary (55°) paraffin. These experiments, of course, refer only to dog serum alkaline phosphatase and not necessarily to other alkaline phosphatases in this or other species.

There remained practically no recognized alternative to fresh tissues on the one hand and acetone or alcohol-fixed paraffin sections on the other, until Seligman, Chauncey and Nachlas (1951) published their observations on formalin fixation. These were derived from efforts to find a suitable fixative for sulphatase and β-glucuronidase, neither of which could tolerate even the moderate heating required by paraffin embedding after acetone fixation. The authors tested homogenates of rat liver for activity in respect of five different enzymes, the homogenates being prepared from liver slices treated for varying times at varying temperatures with 10 per cent. formalin buffered at pH 7 with phosphate. The following Table, modified from Seligman *et al.* (1951), gives the most interesting results of these experiments.

TABLE 3

Percentage Activity Remaining after Fixation in 10 per cent. Formalin

Time	Temperature	Alkaline phosphatase	Acid phosphatase	Esterase	Sulphatase	β-glucuronidase
2 hours . .	4°	73	79	82	74	86
24 hours .	4°	26	55	35	58	87
1 hour . .	25°	48	58	67	59	100
1 hour . .	37°	29	41	27	63	105

The blocks used in the preparation of homogenates, from which the above recordings were derived, were washed for 5 minutes only after exposure to the fixative. The authors found no alteration in results if the tissues were washed for 24 hours. This is perhaps somewhat surprising in view of the observations on the action of formalin described in the opening section of this chapter. The results obtained by Seligman *et al.*, compared with those of Stafford and Atkinson (1948), showed that in the case of both alkaline and acid phosphatases greater activity remained after 24 hours in formalin at 4° than with acetone at 4° and subsequent paraffin embedding. Formalin at higher temperatures destroys enzymes much more rapidly. Reis (1956) has observed that only 22 per cent. of human placental 5-nucleotidase survives 15 minutes' formalin at 20°, and only 4 per cent. survives 24 hours' fixation. For non-specific alkaline phosphatase the figures are 33 and 17 per cent. respectively. These figures differ markedly from those published by Novikoff (1952), who tested survival levels in

thin slices after cold acetone fixation and paraffin embedding. He found 58 per cent. of alkaline phosphatase and 47 per cent. of 5-nucleotidase surviving. For acid phosphatase and adenosine triphosphatase his figures were 41 per cent. and 1·6 per cent. respectively. The contrast between these results serves to stress the vital importance of temperature in the preservation of enzymes.

It is possible to proceed from cold formalin to paraffin embedding, with preservation of a reasonable percentage of some enzymes, provided that dehydration is carried out in acetone rather than alcohol. Such a procedure was used by Gomori and Chessick (1953). It is necessary to remove excess formalin before proceeding to the initial stage of dehydration in cold acetone, and Gomori and Chessick washed their tissues in running water for 24 hours between the two stages. This causes great loss of soluble enzymes, and to reduce these I have substituted 24 hours in cold distilled water (4°). The full routine is recommended only when the particular enzyme which is being studied has been shown to survive adequately. Some of the non-specific esterases of the nervous system and other tissues come into this category.

The employment of short periods of fixation in cold formalin, or formol-calcium, followed by the preparation of frozen sections, must be seriously considered for the histochemical demonstration of all enzymes known to withstand such treatment. Though the method is not perfectly suited to all techniques, even for those enzymes which it preserves adequately, the sharpness of localization which can be obtained and the excellent morphological picture compared with that given by acetone-fixed material should lead to its wider employment. It is particularly suitable for the demonstration of alkaline phosphatase (Chapter XIV), acid phosphatase (Chapter XV) and many non-specific esterases (Chapter XVI). Shorter formalin fixation (4 hours, 4°) is particularly suitable for the β-glucuronidase technique of Friedenwald and Becker (1948), modified by Burton and Pearse (1952), which uses 8-hydroxyquinoline glucuronide as substrate (Chapter XVII), but Fishman and Baker (1956) have recently preferred 20 per cent. formalin containing 0·1 per cent. chloral hydrate at 8° for 17–24 hours.

A comparison of the effect of fixation and embedding processes on various enzymes is given in Table 4 below. The information, representing my own views reinforced by information from other sources (Danielli, 1946 ; Stafford and Atkinson, 1948 ; Seligman *et al.*, 1951), is necessarily incomplete. The percentage of enzyme inhibition quoted by various authors has been converted into percentage of activity remaining.

It can be seen that only in the case of cytochrome oxidase and succinic dehydrogenase is formalin fixation, followed by a frozen section technique, inferior to the other methods listed. In no case does

TABLE 4

Percentage of Enzyme Activity Remaining after Fixation, etc.

Enzyme	Formalin 4° 2–4 hours	Acetone 4° 24 hours	Cold acetone + embedding
Alkaline phosphatase	75	70	30
Acid phosphatase	80	20	6
Non-specific esterase	80	—	*c.* 10
Sulphatase	75	—	0
β-glucuronidase	85	—	0
Choline esterase	10–50*	—	0*–20
Lipase	*c.* 75	—	*c.* 5
Phosphamidase	*c.* 75	*c.* 20	*c.* 5
Cytochrome oxidase	0	traces	0
Succinic dehydrogenase	0	60	0
Tyrosinase (DOPA oxidase)	*c.* 60	—	*c.* 0

* According to some workers specific cholinesterase (AChE) withstands neither formalin nor cold acetone and embedding.

preservation of enzyme after cold acetone fixation and paraffin embedding approach that given by either formalin or acetone fixation alone.

Mucopolysaccharides and Mucoproteins

The fixation of these substances for histochemical purposes needs brief mention only. Holmgren and Wilander (1937) introduced the use of 4 per cent. basic lead acetate as a fixative for acid mucopolysaccharides, when these were to be studied by metachromatic techniques, and Sylvén (1941) used it in combination with formalin. Not all samples of " basic lead acetate " are suitable, but in practice a fresh 4 per cent. solution of pure lead subacetate ($Pb(CH_3CO_2)_2.PbOH_2$), with or without the addition of an equal volume of 10 per cent. formalin, will be found satisfactory. The stock solution of subacetate should be made from CO_2-free water and both this and the mixture with formalin should be protected from atmospheric CO_2. If crystals form in tissues fixed in lead acetate mixtures, they can be removed by brief treatment with 0·1 N-HCl. Other authors have used 1 per cent. lead nitrate in place of acetate, and Lillie (1954) uses an alcoholic 8 per cent. lead nitrate, with or without 10 per cent. formalin for connective tissue mucins of the type found in umbilical cord and vitreous humour. Dziewiatkowski (1953) used formalin saturated with $Ba(OH)_2$ for preservation of mucopolysaccharides containing ^{35}S, and Mota *et al.* (1956) have employed 1 per cent. lead subacetate in 50 per cent. ethanol-acetic acid as a special fixative for mast cells. Although

the above methods give very good results when followed by toluidine blue staining, they are probably not worth while for routine studies of mucopolysaccharides since the latter are quite adequately fixed by formalin, alone or in combination with mercury, and the degree of metachromasia subsequently achieved is often indistinguishable from that seen when lead salts have been employed. Hale (1946) advised the use of Carnoy's fixative for the study of hyaluronic acid by means of his iron method (Chapter IX), and it is certainly adequate for this specific purpose. It should not be used for mucopolysaccharides in general, however.

More recently Williams and Jackson (1956) have introduced two new fixatives for acid mucopolysaccharides. The first is based on a new method of precipitating these compounds with cetylpyridinium salts (Scott, 1955a and b), with which they form highly insoluble complexes. The second depends on the observation that acridines form similarly insoluble complexes. These two fixatives (Appendix 4, p. 789) are certainly superior to formalin and Carnoy, and to formalin-alcohol mixtures for the preservation of mucopolysaccharides. They are not always superior to the lead-containing fixatives, however, but because of their stability and ease of preparation they are to be recommended for all routine and research studies on mucopolysaccharides. Of the two, I prefer acridine-ethanol for most purposes.

For studies of the mucoproteins, exemplified by the anterior pituitary mucoprotein hormones and their precursors, formalin is an essential component of whatever fixative is employed. These materials are very soluble in water and partially in 50 per cent. acetone, 70 per cent. alcohol or 50 per cent. dioxane in water (Fevold, 1937). They are not readily precipitated by picric or trichloracetic acids (Meyer, 1945) but absolute alcohol and acetone cause complete precipitation. After such precipitation, however, the mucoproteins of the pituitary gland are still soluble in water and other solvents. In practice, if alcohol or acetone fixation is followed by paraffin embedding, sections cut from such blocks show complete or almost complete loss of mucoprotein. Formalin, on the other hand, is an inadequate fixative for the mucoproteins of many mammalian pituitary glands. These remain water-soluble unless they are subjected to further precipitation in 70–80 per cent. ethanol for 3–6 days before clearing and embedding in paraffin.

Enzymes as Reagents

The necessary prerequisites for any fixative used to prepare tissues for application of various enzymes are (1) that it should preserve without change, movement or loss, the substrate for the enzyme concerned, and (2) that it should leave the substrate in such condition

as to be readily attacked by the enzyme. Between these two conditions there is a certain degree of antagonism and, on the whole, those fixatives which satisfy the second condition perform indifferently in the matter of the first. Many fixatives exert a profound effect on the subsequent action of enzymes in digesting or removing various structures. This effect is nearly always to delay or prevent the action of the enzyme entirely. In some cases (e.g. diastase), however, no inhibition is produced by a wide range of fixatives. Stowell and Zorzoli (1947) made a study of the fixation of tissues as it affected the subsequent action of ribonuclease upon them. They used sublimate-alcohol, chrome-sublimate, Zenker-acetic, Zenker-formol, Petrunkevitch, formalin, Susa, Carnoy, Bouin, and alcohol and acetone, and found that fixation of ribonucleic acid (RNA) was adequate with all except Bouin- and Susa-fixed tissues. From Carnoy-fixed material, however, the RNA was entirely removed by buffer controls in the absence of enzyme. In spite of this, Kaufmann *et al.* (1950) particularly recommended Carnoy for the preservation of nucleoproteins and other proteins prior to treatment with various enzymes. Other than for RNA or RNA-protein, fixation of proteins by Carnoy is quite adequate preparation for extractive methods employing enzyme solutions. Stowell and Zorzoli observed that Zenker-acetic and Zenker-formol, and also chrome-sublimate, markedly interfered with the action of ribonuclease ; the retention of mercury in the tissues may have a similar inhibitory effect. Chromic, acetic and osmic acid mixtures, such as that of Flemming which was recommended by Kaufmann *et al.* (1948) for the fixation of nucleoproteins, modify protein structures in such a way that subsequent hydrolysis can be achieved only by prolonged digestion with high concentrations of the enzyme.

In most cases formalin is the fixative to which the least objection can be made, although it certainly reduces the action of most enzymes to some extent. An exception is k-toxin collagenase (Chapter XXII, p. 626), whose action is reduced to a minimum by formalin fixation. The alcoholic fixatives and alcohol itself, as we have already seen, leave many proteins in a water-soluble condition especially if the concentration of electrolytes is high. For this reason, even when controls are used, it is sometimes difficult to determine how much of the removal of substrate is attributable to enzyme action and how much to non-specific removal.

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CHAPTER V

PROTEINS AND AMINO-ACIDS

Introduction

In histological preparations proteins are ubiquitous, but nevertheless histologists sometimes wish to demonstrate the presence of protein in a particular site, and to establish the nature of the predominant amino-acids. They may also be concerned with the separation of materials, identified as protein-containing, into one or other of two main divisions of proteins and into one of the several classes in these two divisions. For histochemical purposes the proteins can be divided into (1) simple proteins, defined by the biochemists as yielding on hydrolysis mainly α-amino-acids and their derivatives, and (2) conjugated proteins, which yield substantial quantities of non-protein substances in addition. Some attempt can be made, therefore, to divide protein material in the tissues into the following classes, though the classification in the Table below is neither complete nor entirely accurate from the biochemical point of view.

Table 5

Simple proteins	Conjugated proteins
Albumins	Nucleoproteins
Globulins	Mucoproteins
Albuminoids	Glycoproteins
Globins	Lipoproteins
Histones	

The simple proteins can also be separated into fibrous proteins and globular proteins and the first of these divisions contains the collagens, reticulins, keratin, myosin, elastin and fibrin. These are the constructional proteins, or albuminoids of the older terminology, which are insoluble in most aqueous media. The globular proteins, albumins, globulins, globins and histones are soluble in aqueous media and many of them have been crystallized.

General Identification of Protein

The classical colour reactions for the identification of proteins *in vitro* are violently destructive towards tissues, and many efforts have

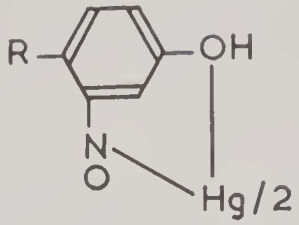
been made to devise innocuous modifications of the original methods. The classical methods which have been employed, with greater or lesser degrees of success, are (1) *Millon's reaction* for tyrosine ; (2) *the diazonium reaction* for tyrosine, tryptophan and histidine ; (3) *the xanthoproteic reaction* for phenolic compounds ; (4) *the Sakaguchi reaction* for arginine ; and (5) *the nitroprusside test* for sulphydryl groups. All these reactions determine only a fraction of the constituent amino-acids of any particular protein, nevertheless a positive reaction has been taken as an indication of the presence of protein since free amino-acids do not occur in tissue preparations from animal sources. All these reactions except the last can be applied to fixed tissue sections, but only two are essentially suitable for the simple identification of protein. They are the Millon and the diazonium reactions.

The Millon Reaction

This reaction (Millon, 1849) is due to the presence in the protein molecule of the hydroxy-phenyl group. It is given by any phenolic compound which is unsubstituted in the position *meta* to the hydroxyl group, but such compounds are not found free in the tissues and the only known amino-acid containing the hydroxy-phenyl group is tyrosine.

The original Millon's reagent was made by digesting mercury in nitric acid and diluting the resulting solution with water. Numerous chemical identities occurred in such a mixture and no explanation of its mechanism was initially forthcoming. Following Hoffmann (1853), Meyer (1864) described the essential features when he produced a positive reaction with tyrosine, $HgCl_2$ and nitrite. Nasse (1901) showed that any phenol gives a red colour after contact with mercuric ions followed by nitrite ions.

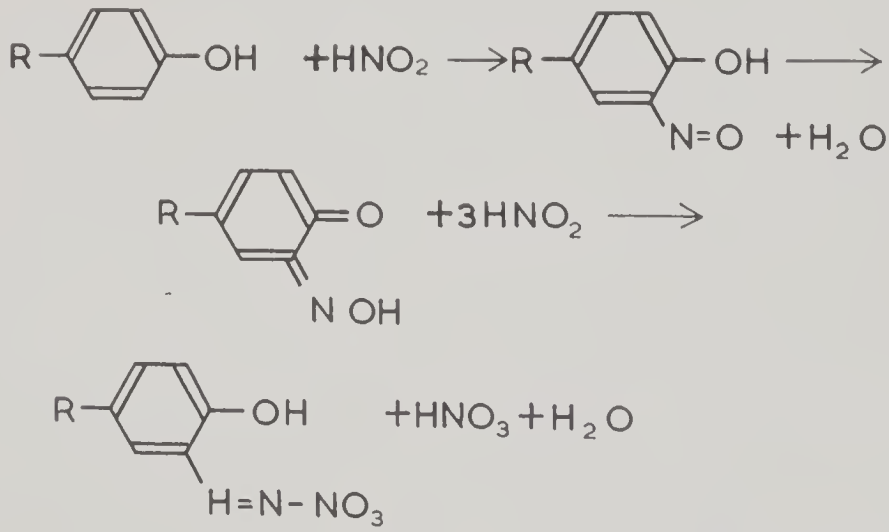
For histochemical use the reagent was modified by Bensley and Gersh (1933) for use in the cold, and employed in the demonstration of mitochondria in freeze-dried sections. If it is used for paraffin sections the application of moderate heat is essential and the warm reagent must be washed off with dilute nitric acid as soon as the maximum colour has developed. This method is given in Appendix 5 (Fig. 28). Other modifications of the Millon reaction for histochemistry include those of Serra (1946) and Baker (1956). The latter is especially recommended for routine use as being simple and reliable and giving more colour, in my hands, than the other reactions cited. Full details appear in Appendix 5. According to Gibbs (1927) the reaction proceeds in two stages. First, a nitrosophenol is produced by the substitution of NO for H *ortho* or *meta* to the hydroxyl of the phenol. Secondly, Hg^{2+} is incorporated into a new ring, by chelation, which includes the nitrogen of the nitroso group. The new complex is red in colour.



I have endeavoured, so far unsuccessfully, to increase the colour of the reaction by treatment with other chelators for mercury. Treatment with diphenylcarbazone, as in the Okamoto reaction (Chapter XI, p. 314), produces a deep purple colour in various tissue components. Blocking reactions, however, show that mercury is present in large amounts in combination with other reactive groups in addition to nitroso-tyrosine.

Morel-Sisley Diazotization Method for Tyrosine

Lillie (1957b) claims that prolonged treatment of sections with cold HNO_2, following the principles given by Morel and Sisley (1927), results in the C-nitrosation of the tyrosine groups of the proteins. The C-nitroso groups are then progressively converted into the quinone oxime tautomer and thence, by further reaction with HNO_2, into diazonium nitrates.

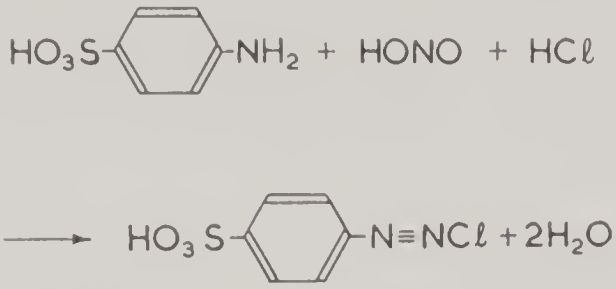


These diazonium nitrates can be coupled with amines in alkaline solution to give coloured reaction products. Lillie uses 1-amino-8-naphthol-4-sulphonic acid for this purpose. It gives a pinkish colour in most structures although hair cortex and keratin stain purplish red. The method (Appendix 5, p. 792) cannot be used as an alternative to the Millon reaction for protein-bound tyrosine unless it can be shown conclusively that tryptophan does not react under the conditions used. According to Eagle (1936) this amino-acid gives a much stronger

reaction with the Morel-Sisley procedure *in vitro* than does tyrosine. Using α-naphthol as final coupler the reaction with tyrosine was insignificant.

The Diazonium Reaction

Diazonium compounds are prepared by the action of nitrous acid in the cold on the salts of primary aromatic amines, sulphanilic acid (*p*-aminobenzene sulphonic acid) being commonly used for this purpose.



The resulting compounds, acting in alkaline aqueous solutions as diazonium hydroxides, combine with the phenol group of tyrosine, the indole group of tryptophan and the heterocyclic imidazole group of histidine to give coloured products. Other amino-acids present in proteins are known to be able to react with diazonium salts to give products which may be coloured in some instances.

A method for the simple demonstration of proteins in tissue sections, making use of the above principle, is the "coupled tetrazonium reaction" of Danielli (1947). This author observed that the diazonium reaction, as usually employed, was relatively useless because compounds of diazonium hydroxides with the amino-acids tyrosine, tryptophan and histidine were only faintly coloured. He therefore substituted for the original diazonium salt a bis-diazonium salt (diazotized benzidine or *o*-dianisidine) and attached a variety of phenols or amines to the free diazo group of the protein-diazonium compound, making a deeply coloured protein-bisazo-phenol in its place. This reaction, which is considered more fully below and in Chapter VIII, is applicable to all kinds of fixed frozen or paraffin sections and also to freeze-dried material. Technical details are given in Appendix 5.

The Xanthoproteic Reaction

This reaction is employed in unmodified form using concentrated nitric acid followed by washing and exposure of the section to the fumes of ammonia. It gives a positive result with tyrosine, tryptophan and phenylalanine, and the presence of these can be identified in what remains of the section by a bright orange colour. The method is too destructive to be useful.

The remaining methods listed at the beginning of this section are not used for confirmation of the presence of protein but only to identify the particular amino-acids for which they are specific. They are referred to below in the section on determination of individual amino-acids.

The Mercury-Bromphenol Blue Method (HgBPB)

This staining method, which is not primarily histochemical, was introduced by Durrum (1950) for the demonstration of protein on filter-paper spots. It was adapted as a general stain for protein by Mazia, Brewer and Alfert (1953). These authors stated that preparations stained by their procedure followed the Beer-Lambert laws and that the amount of dye bound was proportioned to the amount of protein over a wide range. The method was employed by Bonhag (1955) for investigating the composition of the ovary of the milkweed bug *Oncopeltus fasciatus*, and some of his modifications are incorporated in the technical details given in Appendix 5. The histochemical uses of this method are necessarily limited and I have not used it myself.

Physical Differentiation between Classes of Protein

Identification of the simple proteins is achieved by observing their deficiency in those properties which characterize the conjugated proteins. These are subsequently described in the several chapters on conjugated proteins. Some simple protein structures, of particular interest to histologists, are dealt with in Chapter VII.

The chemist separates the various classes of simple protein by making use of their differential solubilities. Albumins, characteristically soluble in pure water ; globulins, insoluble in water but soluble in various salt solutions ; histones, soluble in water but insoluble in dilute solutions of ammonia ; and albuminoids, which are insoluble in all neutral solvents, but soluble in acids and alkalis, constitute the main groups with which the histochemist is concerned. Collagen, elastin and keratin, the so-called scleroproteins, are the chief members of the last group. The globins and histones are known as basic proteins, because they are composed predominantly of the strongly basic amino-acids histidine, arginine and lysine. They differ from each other in that the former contain large amounts of histidine and average amounts of arginine and tryptophan, while the latter contain large amounts of arginine and at most traces of tryptophan. (According to Stedman and Stedman (1947), histones never contain tryptophan.)

The isoelectric points of the various proteins are also significant. At these points many of their physical characteristics, such as solubility, osmotic pressure, viscosity and the property of swelling in water, are reduced to a minimum. The isoelectric point of hæmoglobin, for instance, is between pH 6·7 and pH 6·8, that of serum albumin is

pH 4·88 and that of the globulins between pH 5 and pH 7. The extremes are illustrated by pepsin, whose isoelectric point is below pH 1·0 and the protamines, in whose case it is between pH 12 and pH 12·4. The following methods are used to identify various types of protein.

Simple Solubility Tests

Most of the physical properties which divide albumins, globulins, globins and histones cannot be utilized in the study of tissues fixed by ordinary methods; with freeze-dried material, however, it is possible to make solubility studies which give an approximate identification of the tissue protein. Preferably using sections mounted on slides without contact with water (see Chapter III, p. 43), the solubility of the unfixed protein components can be determined. Water, 1 per cent. NaCl, half and fully saturated ammonium sulphate, dilute solutions of ammonia and various buffers, for instance, are employed as solvents. In this way Gersh (1949) identified the properties of one fraction of thyroid colloid with those of thyroglobulin and Catchpole (1949) similarly identified part of the glycoprotein material in the rat hypophysis with follicle-stimulating hormone. The presence, after various extractions, of residual material giving positive Millon and other reactions may reveal that materials which have been supposed to contain a single protein are in fact composed of several, with widely differing solubilities. This is a method of great promise which should be more widely applied. It must be realized, however, that freeze-dried proteins may differ appreciably in their physical qualities from the original protein of the tissues (see Chapter III, p. 45).

Isoelectric Point by Minimum Solubility (Appendix 5)

The isoelectric points of proteins, *in vitro* and in freeze-dried preparations, can be determined by estimation of their points of minimum solubility in buffer solutions. For freeze-dried preparations a modification of the method used by Catchpole (1949) has been employed. This is applicable not only to the examination of simple proteins, but also to various conjugated proteins. The maximum amount of stainable protein, in the particular site which is being examined, occurs in the sections treated with buffer solutions in the region of the isoelectric point of that particular protein. If the protein is a simple one it can be demonstrated most conveniently by means of the coupled tetrazonium reaction, or by a staining method such as HgBPB; if it is a muco- or glycoprotein the periodic acid-Schiff reaction (Appendix 9) is more suitable, while nucleoproteins are demonstrated by the Feulgen reaction for deoxyribonucleoprotein or by the Brachet test for ribonucleoprotein (Appendix 8).

Isoelectric Point by Staining at Controlled pH

On account of their free basic (amino) and acid (carboxyl) groups, proteins are presumed to behave in solution as amphoteric electrolytes which are capable of entering into chemical combinations with acids and bases to form ionizable salts. The dyes used in staining are either acid or basic and dissociate to form negatively or positively charged ions respectively. Below the isoelectric point of a protein acid dyes combine with positively charged protein ions and above this point basic dyes combine with negatively charged protein ions. Thus any structure in the tissues will be stained by a basic dye if the pH is above its isoelectric point and by an acid dye if it is below that point. Methods for determining the isoelectric point of tissue components by staining in solutions of basic and acid dyes, at various pH levels, are based on this assumption.

Pischinger (1926, 1927) first attempted to determine the isoelectric point of alcohol-fixed tissue sections, mounted on slides. He used toluidine blue as his basic dye, and first Cyanol extra and later Crystal Ponceau as his acid dye. Zeiger (1930) continued Pischinger's work and investigated the effect of different fixatives on the isoelectric point as determined by controlled pH staining. A different point was obtained in each case. Both Pischinger and Zeiger used strong dye solutions at intervals of about pH 0·2 and plotted the staining intensity of the tissue component against the pH of the solution. They took the "mid-point" of their curves for the basic dye as the isoelectric point of the protein. If their technique is to be considered even approximately accurate, the point at which the curves for the basic and acid dye cross should be taken. Up to this time little consideration had been given to the possible effect of varying the dyes and buffers used, and the concentrations of both. Rawlins and Schmidt (1929), however, had already shown that the dissociation of three different dye-protein compounds, though similar from pH 7 to pH 12, differed widely below pH 6. The important work of Levine (1940), conclusively proved that the staining intensity of a protein in buffered dye solutions was dependent on the interaction of the dye-protein, buffer-protein and dye-buffer systems and that as the type of dye and the type of buffer, or the concentrations of either, were altered, the resultant isoelectric points also varied. Levine considered, however, that even if the true isoelectric point was not obtained, the shift of the apparent isoelectric point with different fixatives might well be significant. He introduced a technique in which all the variable factors were reduced to a minimum. The effect of the rate of staining was eliminated by using dilute (M/2000) solutions of dyes in dilute (M/10) buffers and by staining until an equilibrium was reached (24 hours).

To avoid loss, especially of the basic dyes, when passing through the alcohols during the process of dehydration, Levine (1939) introduced the use of tertiary butyl alcohol in place of ethyl alcohol.

As the result of this work we must conclude that staining at controlled pH does not determine the true isoelectric point. Nevertheless, it remains a useful method for differentiating between various tissue components, especially those which exhibit basophilia, and for quantitative expression of alterations in the basophilia of such components, produced by various experimental manœuvres. Differentiation of mucopolysaccharides from mucoproteins, by a method of controlled pH staining, is referred to in the section dealing with those substances (Chapter IX, p. 255). From Levine's data, it appears that methylene blue and orange G should be the most suitable pair of dyes for general use in controlled pH staining and methylene blue proves entirely satisfactory in practice. Orange G, however, fails in my experience to give a decisive end point to many tissue components at high pH values, although it gives a fairly sharp drop in staining intensity between pH 4 and pH 6 when used upon basophilic tissue components.

The indicator dye Solochrome cyanine RS, which I have used as a complexing reagent for aluminium deposits in the tissues (see Chapter XXIV, p. 693) can be used in acid solutions (pH 1·4 to pH 2·1) as a progressive stain for basic and acidic proteins. At these pH levels it stains nucleic acids blue and basic proteins red, while in fresh or fixed frozen tissues acidic lipids also stain blue (Plate IIA, Fig. 92, p. 336). In mammalian and invertebrate tissues the protein component of the mucin takes the stain and shows red, therefore. The acidic (sulphate) groups of most acid mucopolysaccharides apparently have no affinity for Solochrome cyanine RS at acid pH levels. This property is a most convenient one. An exception must be recorded in the case of certain connective tissue mucins, especially those in young connective tissues, which may stain blue rather than red or pink. Changes in the pH at which staining is carried out change red-staining components to blue. The pink (fixed) or red (unfixed) colour of collagen becomes blue at about pH 3.6, for instance. This is not the I.E.P. of collagen, however, which lies between pH 4 and 5. Other red-staining components become blue at different pH levels. The Solochrome cyanine method promises to become a useful addition to the rather limited armamentarium of controlled pH staining.

The apparent differences between the isoelectric points of albumins, globulins and albuminoids are insufficient for the controlled pH method to be of much use for distinguishing them. Nevertheless, it can be used to give evidence of the presence of the strongly basic proteins such as globins and histones. On preparations produced in a standard

manner, in which all variation of fixation, etc., is avoided, the method can be used to register similarity or dissimilarity between two selected materials. Identical behaviour does not indicate identity ; different behaviour indicates difference in structure or composition, but gives no information as to the cause of this difference which is often far from simple. A method for the distinction of basic proteins of the histone type was reported by Hydén (1943), involving staining in a dilute solution of Ponceau 2R, in an acid medium in the presence of a detergent. More recently Sokoloff *et al.* (1951), using Fast green and methylene blue as their acid and basic dyes, attempted to distinguish between various types of fibrinoid and collagen and fibrin. They employed the spectrophotometric method of Singer and Wislocki (1948) and found similar " isoelectric points " in every case, so that they were not successful in determining the origin of fibrinoid.

Identification of Individual Amino-acids

Complete determination of the constituent amino-acids of a particular protein can be achieved by its extraction, purification and the subsequent evaluation of its hydrolysates by chromatography or other methods. Such a manœuvre is beyond the bounds of histochemistry in the restricted sense which is used in this book. Chromatography should, nevertheless, be applicable to the investigation of small quantities of material from freeze-dried preparations, when these have been dissolved in suitable buffer or salt solutions and abstracted from the sections. Thus modern methods of protein analysis, such as those detailed by Springall (1954) in his valuable and instructive book on the structural chemistry of proteins, can be expected to indicate : (1) which amino-acids are present in a given protein ; (2) the percentage amino-acid composition ; (3) the minimum MW of the protein (allowing at least one residue per molecule of the rarest amino-acid present), and (4) the number of amino-acid residues of each sort which go to make up the protein molecule. Modern histochemical methods, on the contrary, can be expected to give simpler and less complete information about a given protein component.

The methods of the previous section will indicate the predominance of acidic groups (aspartic, glutamic, amide-NH_2) or basic groups (arginine, histidine, lysine) in the protein. Methods given in the following section will indicate the presence of α-amino groups (primary amines) and give a relative approximation of their number. This information is roughly parallel to that obtained by demonstrating the acidophilia of a particular component except that acidophilia may be due to primary, secondary or tertiary amines. Table 6, below, will be found useful as an indicator of the range of dissociation of the various ionizable groups in proteins which are responsible for staining

with acid and basic dyes. It also indicates the pH level at which reactions for the various end groups should be carried out.

TABLE 6

pH Range of Dissociation of Ionizable Groups
(After Springall, 1954)

Source	Grouping	$[---R—H]^{n+}$	$[---R]^{(n-1)+}$	pH range
Terminal . .	α-Carboxyl	—COOH	$—CO_2^-$	3–5
Asp. Glut. .	β and γ-Carboxyl	—COOH	$—CO_2^-$	3–5
Histidine .	Imino	^+NH	N	5–9
Terminal . .	α-Amino	$^+NH_3$	$—NH_2$	7–9
Lysine . .	ε-Amino	$^+NH_3$	$—NH_2$	10–11
Tyrosine . .	Phenolic	OH	O^-	10–12
Cysteine . .	Sulphydryl	SH	S^-	10–12
Arginine . .	Guanidino	$C=^+NH_2$	C—NH	13

At strongly acid pH levels (pH 1) all the groups in the table exist in the undissociated $[---R—H]^{n+}$ form, while at strongly alkaline pH levels (pH 14) they all exist in the dissociated form $[---R]^{(n-1)+}$. Each group dissociates over a particular short pH range which is characteristic of that group.

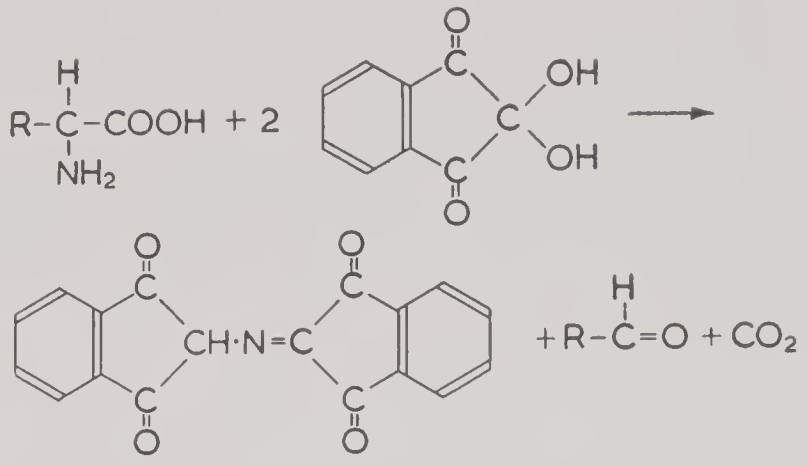
Unfortunately the determination of individual amino-acids in tissue sections is limited by the small number of specific procedures available, by the unsatisfactory nature of many of these and by the complexity of some of the others. The behaviour of the amino-acids in denatured proteins in tissue sections differs from their behaviour in native tissue proteins and, even more, from their behaviour in the test-tube. Since the histochemist deals nearly always with amino-acids in the first group he must expect discrepancies when comparing histochemical with biochemical results. The available methods will be considered under the following headings :

(1) Reactions for amino (NH_2) groups.
(2) Reactions for carboxyl (COOH) groups.
(3) Reactions for tyrosine, tryptophan and histidine.
(4) Reactions for cystine and cysteine (SS and SH groups).
(5) Reactions for arginine.
(6) Blocking reactions for protein end-groups.

Reactions for Amino Groups

Ninhydrin. Of the older methods of histochemistry applicable to the study of amines, the best known is the ninhydrin reaction. Ninhydrin reacts with the free NH_2 groups of α-amino-acids to give a

blue-coloured compound, together with carbon dioxide and an aldehyde containing one less carbon atom than the original amino-acid.



During the reaction, which is carried out at 100°, the bluish-violet colour develops rapidly. This is not stable, however, and, in addition, the blue compound is diffusible in the tissues of the section. Since free amino-acids do not occur in ordinary tissue preparations which have been in contact with water, or in freeze-dried material unless contact with water has been specifically avoided, only the α-amino groups of proteins can possibly react. The number of these available for reaction appears to be small and, in practice, little or no colour develops when the method is applied to tissues prepared by the usual histological techniques. Even the modification introduced by Serra and Queiroz Lopes (1945) using ninhydrin in M/15 phosphate buffer at pH 6·98 gives indifferent results on fixed materials, although it gives a satisfactory dark blue colour with freeze-dried sections. Moore and Stein (1948) have more recently modified the ninhydrin reaction for biochemical use in chromatography of the amino-acids. These authors claim that the colour is improved by using the reduced form of ninhydrin (hydrindantin). They add to the ninhydrin, dissolved in methyl cellosolve (ethylene glycol monomethyl ether), 1·6 per cent. stannous chloride in citrate buffer at pH 5·0. Reduction to hydrindantin occurs and the solution, when diluted, is ready for use. Even though the colour produced by the application of this modification to histochemical use is better than that previously achieved, the extreme diffusibility of the coloured product makes the localization of NH_2 groups by the ninhydrin reaction unsatisfactory.

Alloxan. The condensation of alloxan with amino compounds to give a coloured product is largely a biochemical method. Serra (1946), who quotes Winterstein (1933) as saying that the reaction is given by free NH_2 groups and possibly by SH groups also, found that the test was insensitive with fixed materials and that the coloured product

was diffusible. The alloxan reaction has been criticized by Romieu (1925) on account of lack of specificity and by Giroud (1929) because of diffusion of colour ; Vercauteren (1951) maintained that the reaction product behaved as an anionic dye and stained tissue components having an affinity for such dyes. The alloxan method is, therefore, likely to be of little use in histochemistry.

A number of methods, employing new principles, have been suggested for the localization of NH_2 groups in the tissues. These methods were introduced by Danielli (1947, 1950), and they fall into two separate classes, dinitrofluorobenzene and associated reactions, and azomethine reactions.

DNFB and Isocyanate Methods. These methods depend upon treatment of the tissue sections or smears with either DNFB or *p*-nitrophenyl isocyanate, following the use of blocking agents to prevent the reaction of SH and other groups. Identification of the NH_2 dinitrophenyl compound can be made by its yellow colour, if this is sufficiently strong, and in this case the reaction of DNFB with tyrosine and histidine can be ignored since the resulting compounds are colourless. Alternatively, in the case of both DNFB and *p*-nitrophenyl isocyanate, the NO_2 group may be reduced, diazotized and coupled to a suitable phenol to get the final colour. Table 7 gives the reactions of DNFB and *p*-nitrophenyl isocyanate which can be made the basis of coupling reactions for the histochemical demonstration of the groups listed.

TABLE 7

Reagent	Tyrosine	Histidine	SH	NH_2
Dinitrofluorobenzene .	+	+	+	+
p-nitrophenyl isocyanate .	+	+ or −	+	+

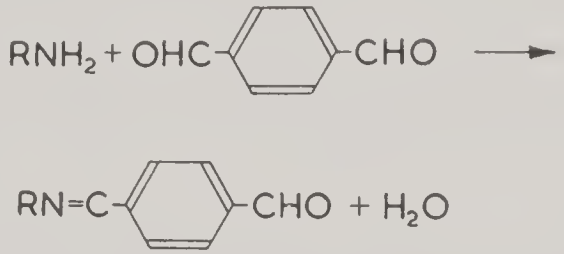
It will be apparent from the Table that unless SH, tyrosine and probably histidine are previously blocked, the reaction with either of the above compounds will not be specific for NH_2 groups. Danielli used treatment with H_2O_2 or iodacetamide to eliminate SH, but made no observations on the elimination of the other groups. I have attempted to get rid of tyrosine and histidine by initial coupling with a diazonium compound, using, for convenience, a stable diazotate of 5-nitroanisidine. Provided nitrous acid is absent from the final alkaline solution of the diazonium compound, only a faint yellow colour is developed with the tissue proteins and their NH_2 groups are presumably unaltered. The DNFB method is considered at greater length in the section below (p. 90) on tyrosine, tryptophan and histidine.

Azomethine Methods. The second class of methods makes use of the formation of azomethines, or Schiff's bases, when aldehydes condense with primary amines.

$$R.NH_2 + OCH.R' \rightarrow R.N = CH.R' + H_2O$$

In tissue proteins these are the ϵ-amino groups of lysine, the δ-amino groups of ornithine, and the α-amino groups of terminal amino-acids.

For the histochemical demonstration of NH_2 groups, Danielli suggested the use of several aldehyde compounds, two of which may be cited as illustrating two different visualizing processes. The first compound, terephthalaldehyde, reacts with tissue NH_2 groups as follows :



and the resulting azomethine is treated with leucofuchsin, with which it gives a red colour by virtue of its unattached CHO group. The second type of aldehyde compound suggested by Danielli is *para*-aminobenzaldehyde. This reacts with tissue NH_2 groups to form an azomethine, leaving a free amino group in the *para* position. This is treated, as previously described, by diazotization and coupling with a suitable phenol.

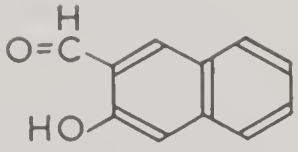
These DNFB, isocyanate and azomethine methods appeared to offer considerable promise for the accurate localization of NH_2. Of those which I tested, however, only the terephthalaldehyde method gave consistent results with conventionally prepared sections. Structures which stained strongly included nuclei, red cells and voluntary and involuntary muscle. Sections in which the NH_2 group had been destroyed by treatment with nitrous acid showed little difference from those in which the NH_2 groups were intact. This applied to unfixed freeze-dried material (Fig. 29) as well as to fixed, paraffin, sections. It is clear that the mechanism of staining is not so simple as suggested by Danielli and that the method is not of value for the detection of NH_2 groups.

Ninhydrin-Schiff and Alloxan-Schiff Methods. These two methods, introduced by Yasuma and Ichikawa (1952, 1953) depend on the demonstration of stable tissue aldehydes which are produced in the course of oxidative deamination with ninhydrin or alloxan (see p. 122). Schiff's reagent is used for this purpose and control sections, not subjected to deamination, should always be employed if there is any doubt as to the

origin of the aldehyde giving rise to the magenta colour of the test section. The mechanism of the two methods is identical and in practice they produce very similar results. I have found, however, that the colour developed with the alloxan variant is often less than that produced by ninhydrin, in parallel sections. Both reactions are blocked by deamination (p. 122) and acetylation (p. 123), applied before the stage of oxidative deamination, and by aldehyde blocking agents (see Chapter XII, p. 341) applied between this stage and treatment with Schiff's reagent. The result of the alloxan-Schiff reaction, applied to paraprotein crystalloids in a granulomatous liver, is shown in Fig. 30, p. 96. Yasuma and Ishikawa specifically stated that 10 per cent. formalin fixation could be employed, as indeed it can, so that in this case formaldehyde blockade of NH_2 groups is apparently reversible.

Chloramine-T Methods. An oxidative deamination method using Chloramine-T (sodium *p*-toluene sulphochloramine) or hypochlorite was suggested by Chu, Fogelson and Swinyard (1953). The tissue aldehydes were demonstrated by means of Gomori's hexamine silver reagent (Appendix 12, p. 863). Chilled alcohol was found to be the best fixative and formalin was purposely avoided. Burstone (1955) tested the oxidizing capacity of chloramine-T, and of sodium and calcium hypochlorites, finding the last to be only one-third as strong as the other two. He was able to obtain stable aldehydes in the tissues only with chloramine-T oxidation, and these were demonstrated with Schiff's reagent. The reaction was blocked by deamination, but after acetylation the nuclei would still react.

3-Hydroxy-2-Naphthaldehyde. A different type of azomethine reaction was proposed by Weiss, Tsou and Seligman (1954) for the demonstration of protein-bound NH_2 groups. Their reagent, 3-hydroxy-2-naphthaldehyde

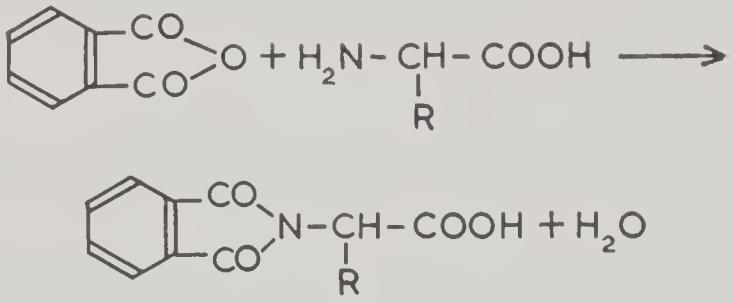


can be prepared from 3-hydroxy-2-naphthoic hydrazide (Appendix 8, p. 823). It is poorly soluble in water and is used, therefore, as a buffered solution in aqueous acetone (pH 8·5). Below this pH level the formation of the pale yellow Schiff's base is considerably reduced. To develop the final colour a second stage is necessary, in which the Schiff's base is coupled with tetrazotized di*ortho*anisidine at pH 7·4. This reaction can be abolished by prior acetylation or deamination and its specificity is therefore high. Fig. 31, p. 96, shows the staining of NH_2 groups by this method in formalin-fixed tissue. The original authors noted that formalin suppressed the reaction, and this is true

for the majority of tissue components. It is evident, however, that structures containing a large number of available NH_2 groups may still react strongly despite formaldehyde blockade.

Details of this method and of the oxidative deamination methods appear in Appendix 5, p. 794.

***O*-Diacetylbenzene.** This reagent was originally used for the histochemical demonstration of α-amino-acids by Voss (1940) and later, for the same purpose, by Dietz (1942). More recently the method has been thoroughly investigated by Wartenberg (1956). The mechanism of the reaction has not been explained, but Winkler (1948) suggested the possibility of a parallel with the formation of phthalocyanins. The equation below shows the condensation of phthalic anhydride with an amino-acid to form a phthalimido acid :



Analysis of the coloured reaction product of the condensation of *o*-diacetylbenzene with ammonia showed that it had a molecular weight of 700 and contained one nitrogen atom.

Wartenberg used *o*-diacetylbenzene as a 2 per cent. solution in 70 per cent. ethanol and found that the reaction was positive in a reasonably short time only if the pH was higher than 8. At this pH level 30 minutes at room temperature produced a deep lilac colour in the sections. The specificity of the reaction is apparently high, since Wartenburg found that it was abolished by acetylation, deamination, benzoylation and by 16 hours' treatment with DNFB. It was weakened, though not reversed, by formalin fixation and reversed by 5 hours' treatment with ninhydrin though not by alloxan for a similar period. Wartenberg claimed, therefore, that *o*-diacetylbenzene reacted only with free NH_2 groups, and it is apparent from his tabular data, and from a study of Table 6, p. 84, that the pH range of the reaction is precisely that at which the dissociation of α-amino groups from $—NH_3$ to $—NH_2$ takes place.

Details of the *o*-diacetylbenzene method are given in Appendix 5, p. 795.

Reactions for COOH Groups

Methyl Ketone Method. Following the observation by Wiley (1950) that the COOH groups of acylamino-acids could be converted

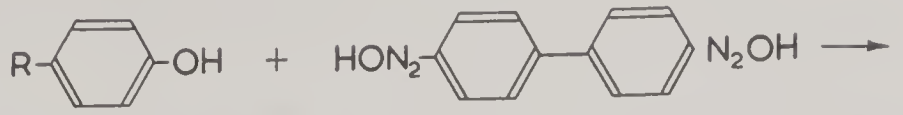
into amido ketones by treatment with acetic anhydride in pyridine, Barrnett and Seligman (1956, 1958) developed a method for the detection of COOH groups in protein. They used paraffin sections fixed in a variety of fixatives, Carnoy being superior to most others, and demonstrated the ketone by means of the Ashbel-Seligman reagent, 2-hydroxy-3-naphthoic acid hydrazide (Chapter XII, p. 338 ; Appendix 8, p. 823). The reaction was completely prevented by previous methylation of the sections. It appears that only terminal carboxyls having an adjacent α-carbon with an electrophilic N group are capable of reacting. The free carboxyls of glutamic and aspartic acids cannot do so. On theoretical grounds one would not expect much colour with this method since the number of available reactive groups in the proteins is necessarily small. The fact that a good colour is developed in a number of acidophilic tissue components is difficult to understand on the basis of a concentration of terminal COOH groups.

Reactions for Tyrosine, Tryptophan and Histidine

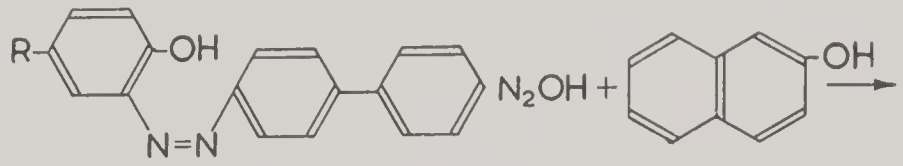
Millon Reaction for Tyrosine. This method has already been fully considered (p. 76) as a general reaction for proteins. Its specificity for tyrosine is absolute, as far as mammalian tissues are concerned, although *in vitro* tryptophan also gives a red colour. Pollister and Ris (1947) used the reaction for quantitative spectrophotometry, employing as a " blank " sections treated by the Millon routine without nitrite. They observed that the absorption maxima of the nitroso-mercury-tyrosine compound were 3,550 Å (U.V.) and 4,800 Å (visible). Rasch and Swift (1953) concluded that the Millon reaction should be used for spectrophotometry only if certain conditions, which they specified, were fulfilled. Broadly speaking the method was found useful only for comparing similar proteins.

Tetrazonium Methods. Observations have already been made, earlier in this chapter, on the use of the coupled tetrazonium reaction of Danielli for the demonstration of protein by virtue of its contained amino-acids, particularly tyrosine, tryptophan and histidine. This reaction is based on the combination of amino-acids with tetrazotized benzidine at pH 9·0 and at 4°, and the subsequent coupling of the residual free diazo group with a phenol or aromatic amine. The following formulæ express the reaction as it takes place with the aromatic hydroxyl group of tyrosine.

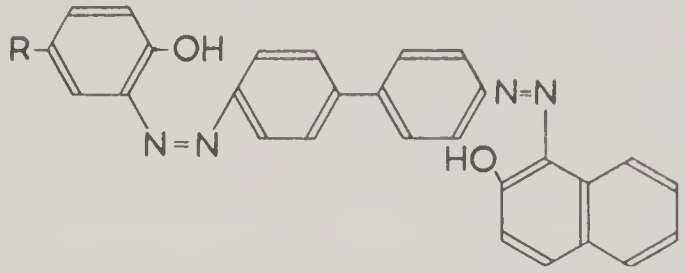
It has been suggested (Pauly, 1904 ; Gomori, 1952) that diazonium salts will not react with tryptophan, but this view is contradicted by many protein chemists. Frazer and Higgins (1953), for instance, studied the reaction of diazosulphanilic acid with tryptophan, tyrosine and histidine. They showed that ring azo derivatives were formed with the imidazole group of histidine and with the phenolic group of tyrosine.



Protein with tyrosine group — Tetrazotized benzidine



Brown compound
(protein-diazonium)



Bright red compound
(protein-bisazo-naphthol)

Azotryptophan apparently lacks colour, but this fact has no bearing on the production of a coloured final reaction product in the coupled tetrazonium reaction.

Zahn, Wollemann and Waschka (1953) found that diazotized 5-chloro-2-methylaniline would react with proline to form a triazene, and with cysteine to form a diazomercaptan, and Gelewitz, Riedeman and Klotz (1954) showed that the reaction of serum albumin with diazosulphanilic acid produced only relatively few reactions with side chains, of which only a third were —N=N— bridges. They suggested that the ϵ-amino groups of lysine could react, but Frazer and Higgins (*loc. cit.*) denied this possibility.

According to Howard and Wild (1957) the reaction of proteins with diazonium salts certainly occurs with other groups as well as those of tyrosine and histidine. They showed that 2 mols. of diazonium salt combined with phenolic and imidazole groups, and with the ϵ-amino groups of lysine. One mol. of diazonium salt combined with cysteine (SH) and tryptophan (indole) and 3 mols. with arginine (guanidino).

In view of these findings, particularly those of Howard and Wild, it is necessary to modify our views of the specificity of the coupled tetrazonium reaction for tyrosine, tryptophan and histidine only, and to abandon the reaction entirely as a method for these amino-acids individually, following the blocking techniques suggested by Danielli. My present view is that the coupled tetrazonium reaction can be used, properly, in two ways only. First, as stated above, as a general reaction

for proteins, and secondly, following benzoylation (p. 127), as a general reaction for proteins capable of undergoing thermal shrinkage. (See, however, Chapter VIII, p. 190.)

Burstone (1955) has emphasized the difficulty of interpreting the results of the coupled tetrazonium reaction. He found in a series of *in vitro* experiments that non-protein substances like filter paper were stained brown by the breakdown products of the tetrazonium salt (the stable bis-diazotate Fast Blue B salt) and noted that even after thorough washing a final coupling with H-acid would produce a deep purple colour. This evidence suggested that some of the colour developed by the reaction might be due not only to amino-acids other than tryptophan, tyrosine and histidine, but to entirely unspecific causes. Using freshly diazotized benzidine I have not found that the colour of either tissue sections or filter paper is other than pale yellow, and, after thorough washing and coupling with H-acid, the latter does not produce a purple colour.

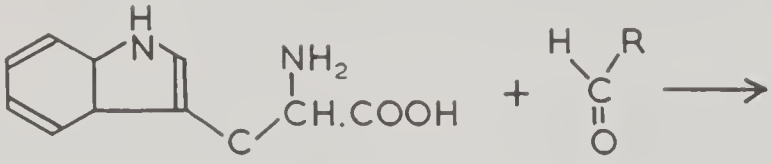
In spite of its restricted usefulness details of the coupled tetrazonium reaction are given in Appendix 5, p. 795.

Dimethylaminobenzaldehyde (DMAB) Methods for Tryptophan. There are at least four biochemical tests for tryptophan : Hopkins-Cole (glyoxylic acid), Voisenet-Rhode or May-Rose (DMAB), Voisenet-Fürth (formol-nitrate) and the Romieu reaction (Blanchetière and Romieu, 1931), employing orthophosphoric acid. The first three are all variations of the historical aldehyde procedure for tryptophan. All these reactions involve the use of strong acids and they are therefore somewhat destructive to the tissues. A modification of the Voisenet-Fürth reaction was reported by Serra and Queiroz Lopes (1945) to be applicable to formalin-fixed paraffin sections, but I found that even when direct mounting in glycerine was employed, as suggested by the original authors, the colour developed was insufficient to allow accurate localization of tryptophan in the tissues. A modification of the May-Rose (1922) reaction, introduced by Bates (1937) and employing sodium nitrate as an oxidizing agent, was used by Kaufmann *et al.* (1946, 1947) in their study of chromosomes, to distinguish histones from tryptophan-containing proteins. I found the amount of colour produced by this reaction similarly inadequate.

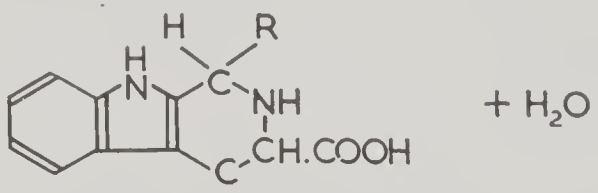
Lillie (1956) applied a modification derived from Feigl (1954) using 2·5 per cent. DMAB in a 1 : 3 mixture of concentrated HCl and acetic acid. He observed that in formalin-fixed tissue the reaction was too insensitive to react with compounds other than those containing large amounts of tryptophan. A positive reaction was obtained with pancreatic zymogen granules which have been shown by Marshall (1954) to contain chymotrypsinogen. This enzyme contains 5·4 per cent. of tryptophan.

The DMAB-nitrite Method. A further modification by Adams (1957a) reintroduced, this time successfully, the use of an oxidizing agent to develop the colour of the final product. Following the work of Spies and Chambers (1948a, 1948b) and Portner and Högl (1953), Adams used brief oxidation with sodium nitrite. This method produces an intense blue pigmentation in tissue components containing much tryptophan (fibrin, fibrinoid, Paneth cell granules, chief cell granules, zymogen granules (Fig. 32, p. 96), thyroid colloid, muscle fibres, neurokeratin and inner hair-root sheath). A moderate reaction is given by liver parenchymal cells, keratin (after alkaline hydrolysis) and the deeper layers of the epidermis, the cytoplasm of many neurones and by the cytoplasm of the cells of the peripheral parts of argentaffin carcinomas. The argentaffin (enterochromaffin) cells (Chapter XXIII, p. 649) do not react ; collagen, reticulin, oxyntic cells, goblet cells, melanins and lipofuschins are similarly negative.

In *in vitro* tests Adams found that only 3-indolyl derivatives would form the blue pigment ; those which he tested were tryptophan, tryptamine, 5-hydroxytryptamine, and 3-indolyl acetic acid. Indole itself formed a greyish-violet pigment after prolonged oxidation. These results confirm those of Spies and Chambers and of Portner and Högl in suggesting that the DMAB-nitrite reaction takes place in two stages. Adams suggests the following mechanism:



Tryptophan Aldehyde

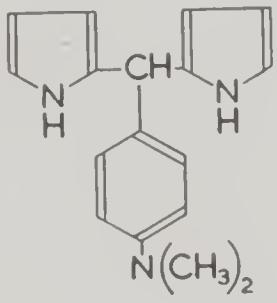


β-carboline

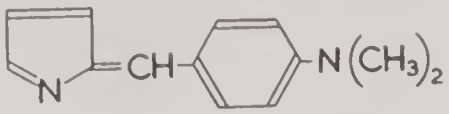
The blue pigment (carboline blue), whose structure is apparently unknown, is derived from the β-carboline by nitrite oxidation. This concept is supported by the observations of Harvey, Miller, and Robson (1941), who produced such a pigment from the colourless condensation product of tryptophan and formaldehyde (2,3,4,5-tetrahydro-β-carboline-4-carboxylic acid, but it fails to explain the formation of a blue pigment from 3-indolyl derivatives having too few C atoms to complete the third ring of the β-carboline. The product of DMAB-tryptophan condensation *in vitro* has a red colour, but in the tissues this

is seldom seen. I found it only in Russell bodies and in the layer of Huxley and Henle in hair sheaths. The specificity of the Adams DMAB-nitrite reaction is high. Although other amino-acids will react with DMAB through their α-amino groups to form Schiff's bases (p. 122) the products are yellow or orange, never red, and they cannot be oxidized to blue pigments. The reaction is entirely prevented by previous oxidation of the tissues with performic acid (p. 125), and also by alkaline persulphate (p. 126), and it is reduced by both chromate and formalin fixation. In the last case interference is slight except with prolonged fixation (e.g. 14 days in 4 per cent. formaldehyde). Technical details are given in Appendix 5, p. 797.

The Rosindole Reaction. Glenner's (1957) rosindole reaction is essentially the same as Adam's DMAB-nitrite reaction except for the use of a mixture of acetic and perchloric acids, containing only a small amount of HCl, as solvent for the DMAB and of acetic-HCl as solvent for the nitrite (Appendix 5, p. 798). The results, in so far as I have compared the two reactions, show an identical distribution of colour. Glenner, however, considers that his rosindole reaction is a benzylidene ($C_6H_5CH=$) condensation of the type postulated by Fischer (1911, 1932). According to this author's views pyrrole compounds condense with Ehrlich's reagent in two ways. Either two pyrroles condense with one aldehyde to give a red dyestuff (I), or one pyrrole condenses with one aldehyde (II), again giving a red dye.



I



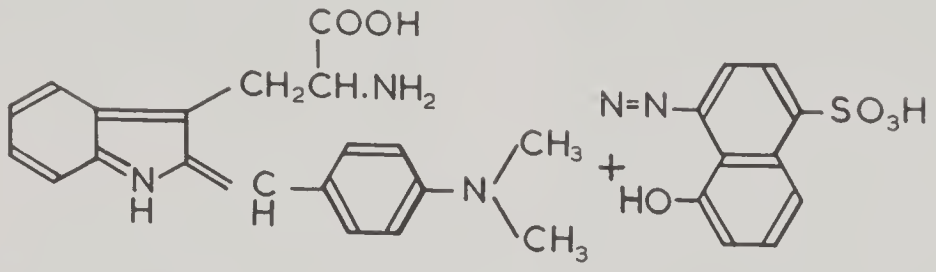
II

Goessner (1947) has shown that the substance in normal urines which gives a positive reaction is indoxyl and that the condensation which takes place belongs to Fischer's type II. Glenner postulates that in the presence of perchloric acid a molecule of tryptophan condenses with a molecule of DMAB to form a phenylindolyl-methane compound, of Fischer's type II, which is in equilibrium with a leuco compound having a structure analogous to Fischer's type I. Oxidation of the latter produces a stable blue rosindole dye. The histochemical rosindole reaction can be produced by aromatic aldehydes other than DMAB and alternative oxidizing agents can be employed. Among the latter, tested by Glenner, are $KMnO_4$, $FeCl_3$, $K_2Cr_2O_7$ and

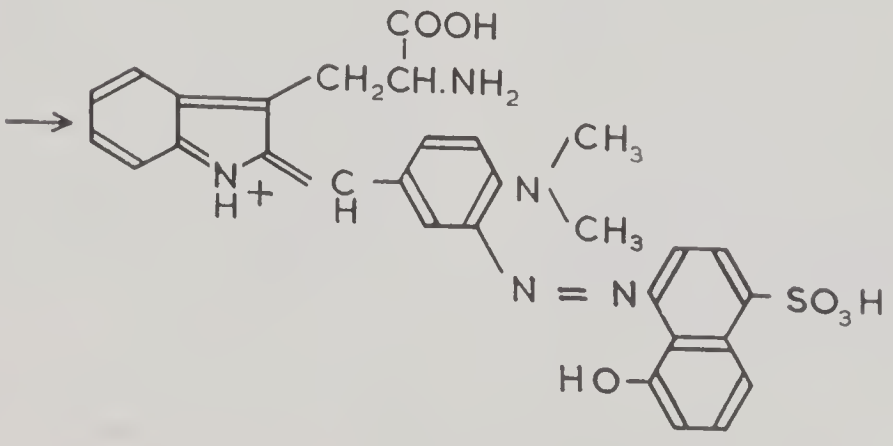
$K_3Fe(CN)_6$. No combination of alternatives produces any improvement over the standard reagents in the rosindole reaction.

I agree with Glenner that the rosindole reaction is more sensitive than the xanthydrol reaction (see below, p. 96) and that the product is more easily visible. I find, however, that the DMAB-nitrite procedure consistently gives more colour than does Glenner's perchloric acid variant. Although this author refers in his Tables to the positive staining of mucous goblet cells, Adams (1957a) failed to obtain this result, and I cannot reproduce it with either of the methods in mammalian or invertebrate tissues. Since tryptophan can be shown to be present in the mucins derived from these cells it is likely to be present in the goblet cells themselves. Failure to show it must be ascribed to stereochemical factors which interfere with condensation.

The Post-coupled Benzylidene Reaction for Tryptophan. This reaction (Glenner and Lillie, 1957) is a variant of the original DMAB reactions in which no oxidation step is employed. In place of oxidation the sections are treated with a freshly diazotized amine salt in acetic acid. The original authors tested several compounds and obtained their best results with S-acid (8-amino-1-naphthol-5-sulphonic acid), which gave a stable dark blue product in the tissues. It is assumed that under acid conditions coupling takes place *ortho* to the free dimethylamino group of the side chain of the phenylindolylmethane compound :



Tryptophan-DMAB Diazotized S-acid



Blue azo dye

When benzaldehyde was substituted for DMAB and followed by diazotized S-acid, no increase in colour was produced. Where the same initial procedure was followed by HNO_2 oxidation a dark blue colour developed. This shows that in the post-coupled benzylidene reaction azocoupling certainly takes place.

Glenner and Lillie found that it was possible to couple diazotized S-acid *ortho* to the free amino group of the oxidation product of the rosindole reaction, with some increase in colour. It is not possible to increase the colour of the DMAB-nitrite reaction by this means. The post-coupled benzylidene reaction is to be regarded as entirely specific for indole derivatives. In tissue sections the only reacting compounds likely to be present are tryptophan, tryptamine and 5-hydroxytryptamine, and these give a strong reaction *in vitro* which is slowly reversed by prior treatment with formaldehyde.

The list of tissue components demonstrated by this method is substantially the same as with the two methods described above, but there are a few outstanding points of difference. In particular, by the post-coupled benzylidene and rosindole reactions human pancreatic α-cells are clearly demonstrated (Fig. 33, p. 96), but with the DMAB-nitrite method they are much less well shown. The original authors reported their inability to block the reaction by previous treatment of sections with performic acid, but in my hands this reagent, made according to the instructions given in Appendix 5, p. 805, is quite successful in blocking subsequent reactions of the tissues with DMAB. Alkaline persulphate oxidation has the same effect. Technical details of the method are given in Appendix 5, p. 798, but I consider that for most purposes the more simple DMAB-nitrite method is preferable.

Two further histochemical reactions for tryptophan must be considered. The first of these is the xanthydrol reaction.

Xanthydrol Reaction for Tryptophan. A method for the determination of tryptophan in proteins using xanthydrol in HCl was described by Dickman and Crockett (1956). This was based on earlier work by Illari (1938) and Fearon (1944). These authors showed that pyrroles would react with xanthydrol in acetic acid, giving purplish-red products. According to Fearon the reaction could take place with indoles only if the 3-position was open, and thus tryptophan did not react. Dickman and Westcott (1954), on the other hand, found that xanthydrol in HCl/acetic acid mixtures gave a purple product with both tryptophan and tryptamine.

The Dickman and Crockett method was adapted by Lillie (1957a), and independently by Adams (1957b) for histochemical use. It gives a violet colour with pancreatic zymogen granules in several species, and also with gastric chief cells, salivary duct cells, and in the fibres of the crystalline lens (Lillie). Smooth muscle, cardiac muscle and

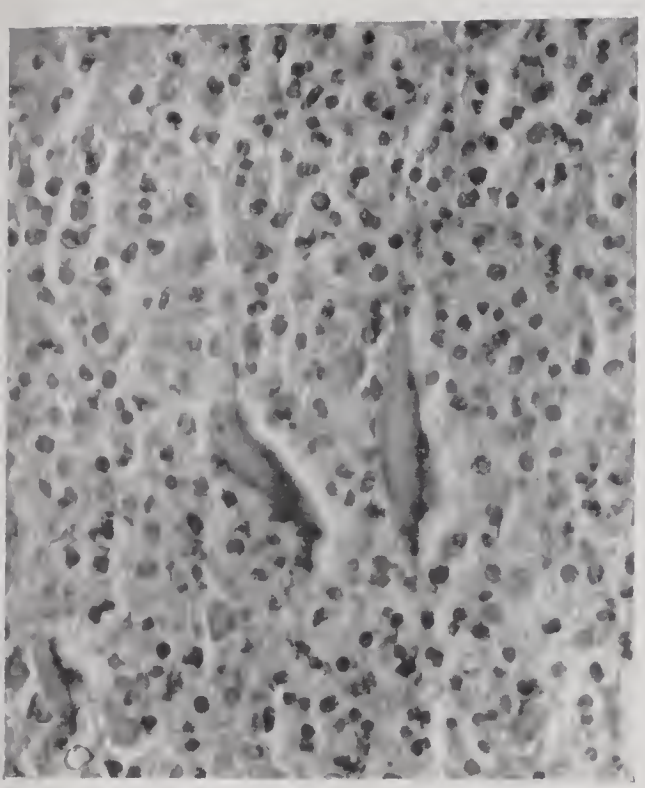


FIG. 30. Human liver. Paraprotein crystals in a granuloma. Alloxan-Schiff. × 510.

FIG. 31. As Fig. 30. Hydroxy-naphthaldehyde method. × 510.

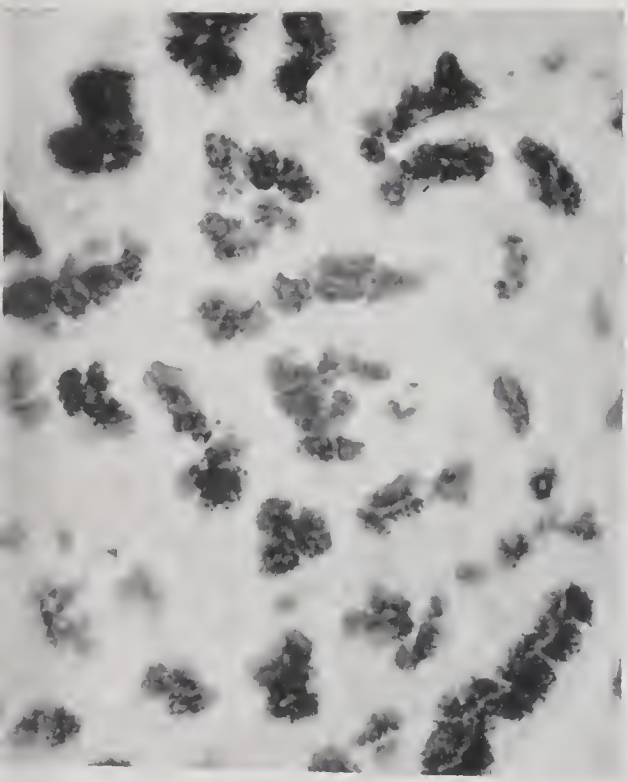


FIG. 32. Mouse pancreas. Intense staining of zymogen granules in the acinar cells. Adams' DMAB-reaction. × 510.

FIG. 33. Rabbit pancreas. Alpha cells in an islet of Langerhans. Post-coupled benzylidene reaction. × 850.

[*To face p.* 96.

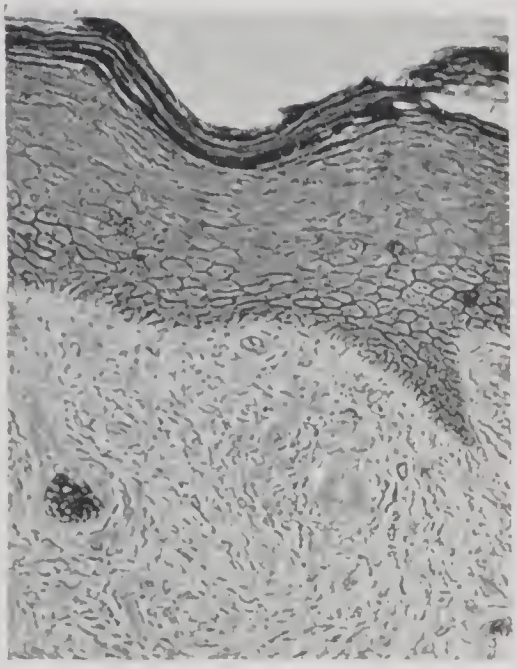


FIG. 34. Human skin, formalin-fixed, 15 μ frozen section. The darker structures contain sulphydryl groups; lower left, red cells in a small artery. Bennett RSR method. × 105.

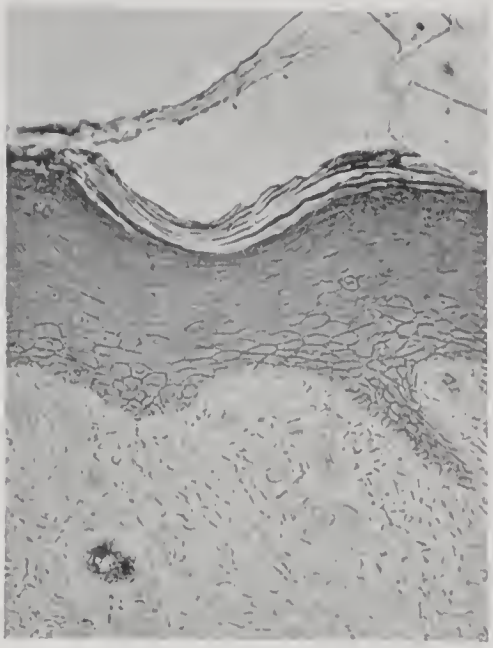
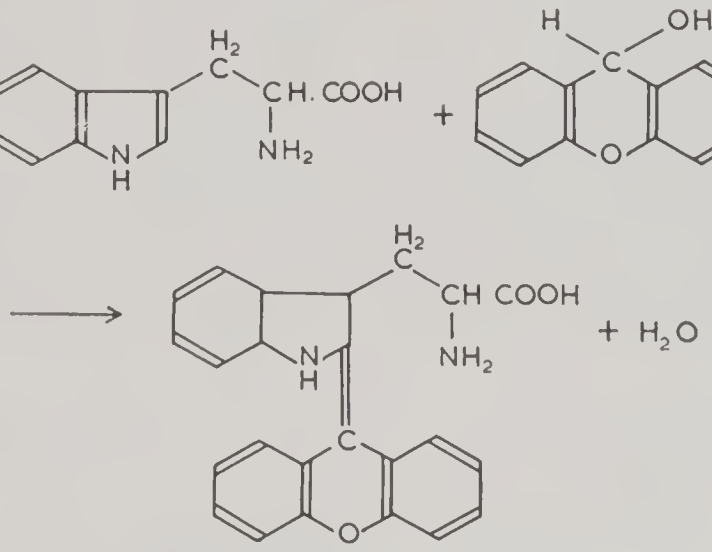


FIG. 35. Similar section to Fig. 34, for comparison. Note the more darkly stained stratum corneum. Chèvremont method for sulphydryls. × 105.

FIG. 36. Longitudinal section of human hair follicle. The outer portion of the hair shaft (cortex and medulla) stains bright magenta. Huxley's layer of the root sheath also stains. Performic acid-Schiff. × 120.

FIG. 37. Transverse section of human hair and root sheath. The cortical layer of hair is stained dark blue, the medulla to a lesser extent. No other structure is stained. Performic acid, M/2000 methylene blue at pH 2·6. × 300.

skeletal muscle fibres give a moderate reaction, goblet cells in the rabbit were just faintly coloured in the original author's hands, but most mucins are negative, as are the argentaffin (enterochromaffin) cells in three species. As with the DMAB-nitrite reaction, some areas in argentaffin carcinomas (carcinoids) contain cells with a moderately strong reaction in their cytoplasm. Lillie (1957a) suggested two possible mechanisms to explain xanthydrol-tryptophan condensation. The one given below differs from the other only in ignoring the part played by HCl.

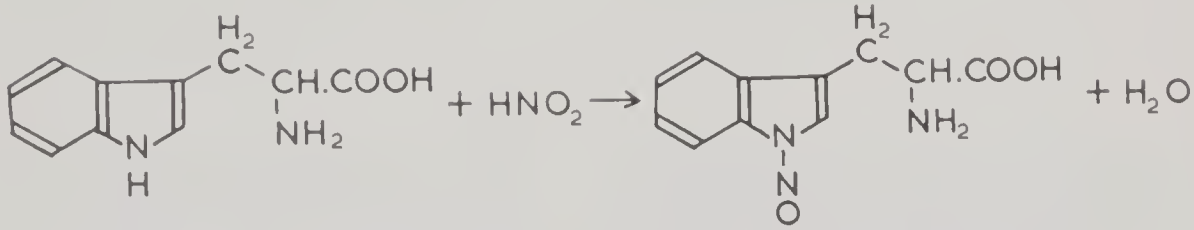


In vitro tests which were made by Lillie gave a positive result with tryptophan, tryptamine, indole, skatole and pyrrole. A dark blue-green colour developed with 5-hydroxytryptamine and a deep blue with carbazole. The latter, incidentally, does not react with DMAB. Strong purplish-black colours were obtained with 2-phenyl and 2-methyl-indoles, 2-phenyl and 2-carbethoxy-tryptophan, and with tetrahydronorharman, pyrogallol, catechol and resorcinol. According to Lillie the reaction was not abolished by treatment of sections with performic acid in the cold. In my hands, using this reagent at room temperature, blocking was complete in 2 hours. The reaction is also blocked by peracetic acid, but benzoylation (p. 127) weakens it only moderately. Iodination with 10 per cent. iodine in methanol (p. 130) also completely prevents the reaction.

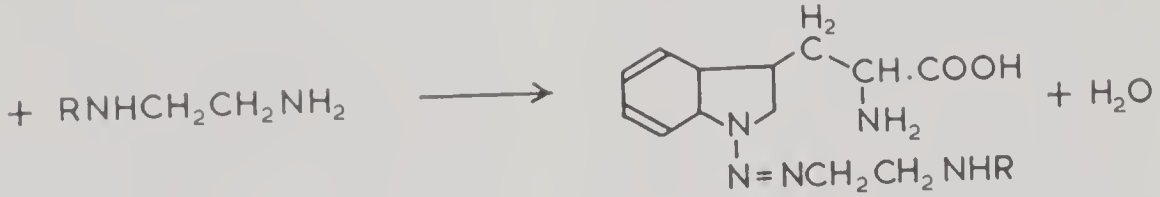
The specificity of the xanthydrol method for tryptophan in tissue sections must be regarded as high, but the colour developed, in my hands, is always less than that given by the DMAB-nitrite reaction, which I regard as the more satisfactory of the two. This reaction possesses an additional advantage in that the colour is stable over a long period (years), whereas that of the xanthydrol method fades in a few months.

The Naphthyl-ethylenediamine (NED) Condensation Method for Tryptophan. An entirely different principle has been employed by Bruemmer, Carver and Thomas (1957) in developing a method for tryptophan from the method of Bratton and Marshall (1939) for sulphanilamides. The reagents used by these authors were adapted by Eckert (1943) to produce a red colour with tryptophan.

With the NED condensation method tissue sections are first treated with nitrous acid and then placed in a solution of N-(1-naphthyl)-ethylenediamine dihydrochloride. A violet colour develops in sites containing large amounts of tryptophan. The mechanism of the reaction is not so simple as appears at first sight. Primary aromatic amines are diazotized by treatment with HNO_2, as in the original method of Bratton and Marshall, and they can then be coupled with phenols or aromatic amines to form azo dyes. Indoles cannot be diazotized, however, and Bruemmer *et al.* suggest that treatment with HNO_2 produces an N-nitroso compound (cf. Baker's explanation of the Millon reaction, p. 76), and that condensation takes place to link the N of the nitroso group with the free NH_2 group of NED :



and the nitroso compound then condenses with the amine



Bruemmer *et al.* found, by infra-red absorption spectroscopy, that the NED-tryptophan condensation product possessed the specific absorption band in the region of 6·3 mμ, which is due to the —N=N— configuration.

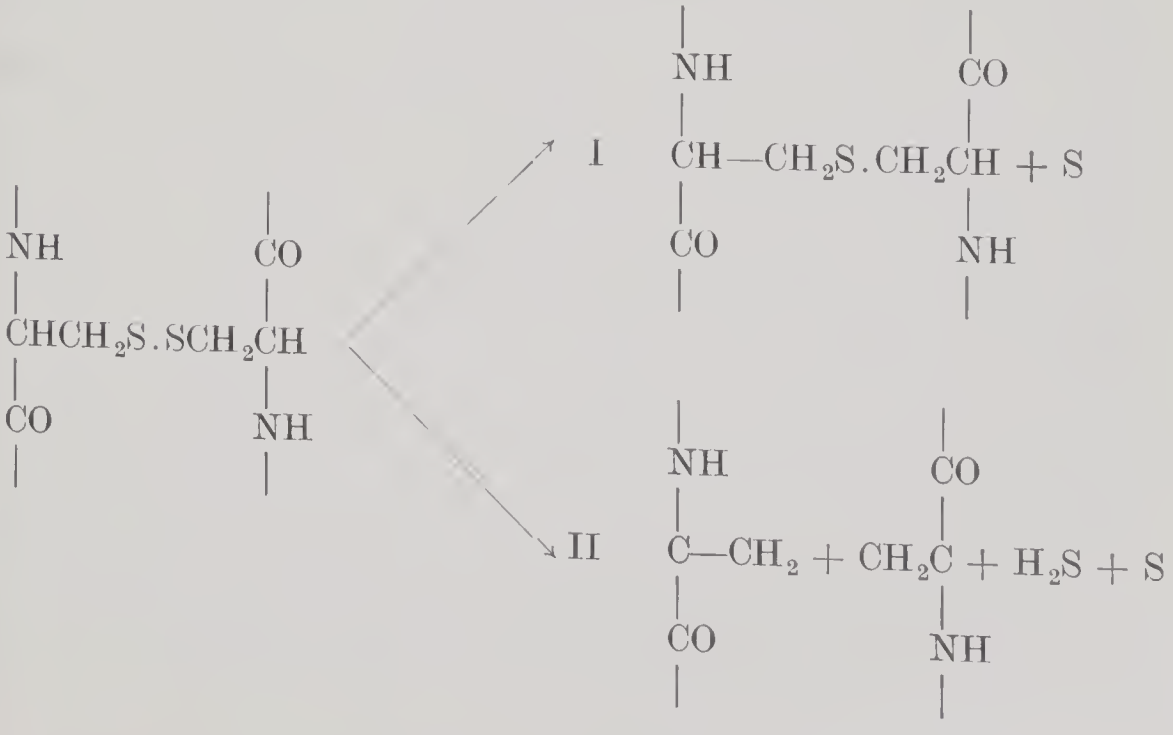
The original authors carried out specificity tests on a number of compounds *in vitro* and obtained a positive result only with tryptophan, skatole, indole acetic acid, indole butyric acid and isatin. Pyrrole and catechol did not react. No blocking reactions were applied to the histochemical reaction, but the NED condensation in the tissues is abolished by both iodination and by treatment with performic acid. Although the specificity of the reaction thus appears to be high enough

thc colour developed is only moderately satisfactory, and I consider that further development is necessary before the method can be recommended for the routine investigation of tryptophan-containing proteins. Details of the method are given in Appendix 5, p. 799.

Reactions for SS and SH Groups (Cystine, Cysteine)

Histochemical methods for revealing the presence of sulphur-containing amino-acids can be divided into eight groups. First, the older lead and nitroprusside methods and their modifications ; secondly, the phenacyl halide methods suggested by Danielli (1950) employing either specific halogen-containing reagents or blocking techniques followed by dinitrofluorobenzene ; thirdly, the method of Bennett (1948) employing organic mercurials ; fourthly, the ferricyanide method of Chèvremont and Fréderic (1943) ; fifthly, performic acid oxidation methods (Pearse, 1951 ; Adams and Sloper, 1955) ; sixthly, the alkaline tetrazolium methods (Barrnett and Seligman, 1954 ; Pearse, 1954) ; seventh, the dihydroxy dinaphthyl disulphide (DDD) method (Barrnett and Seligman, 1952) ; and lastly, the α-naphthyl maleimide method (Seligman, Tsou and Barrnett, 1954).

The Lead Acetate Method for SS and SH. The principle of this method, adapted from the biochemical test for cystine, is the liberation of sulphur from the tissues by the action of strong alkalis at high temperatures and the subsequent demonstration of this as black sulphide of lead. Alkalis hydrolyse and decompose disulphide cross linkages. Cuthbertson and Phillips (1945) showed that wool cystine breaks down in two ways when treated with sodium hydroxide. Approximately 50 per cent. is converted into lanthionine and sulphur (I) and the remainder is decomposed (II).



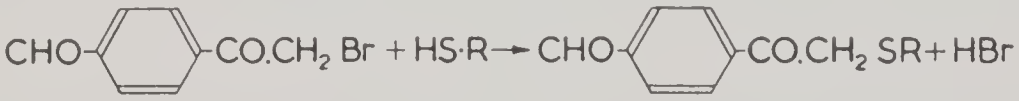
Even dilute (0·1 N) NaOH acts slowly in this manner while more concentrated solutions of NaOH (1·0 N) or barium hydroxide (0·385 N), acting for 1 hour at room temperature, produce a complete result, 50 per cent. conversion to lanthionine and 50 per cent. decomposition.

Mann (1902) observed that since the reaction with concentrated solutions of alkali necessitated almost total destruction of the tissues, its application to histochemistry was not of much use. Nevertheless, using hot concentrated solution of alkali, it is possible to demonstrate the presence of sulphur in keratin at some stage before disintegration of the tissue occurs. The distribution of black lead sulphide is patchy, however, since sulphur is soluble in alkaline solutions, and its localization cannot be accepted as accurate. Theoretically, it should be possible to use more dilute solutions of alkali and still reveal the liberated sulphur *in situ*, unless this has been dissolved away. In practice, I have found that the desired blackening of known sulphur-containing tissues fails to occur when 10 per cent. lead acetate is applied to alcohol or formalin-fixed tissues after the use of alkalis (0·1–2·5 N NaOH) for various times (1–16 hours) at various temperatures (20°–60°). This method must be regarded as useless and of historical interest only.

Nitroprusside Methods for SH. There are numerous histochemical modifications of the nitroprusside reaction for SH compounds, all of which rely on the production of a purplish-red colour when SH groups come into contact with sodium nitroprusside in the presence of hydroxyl ions (usually NH_4OH) and saturated ammonium sulphate. Nothing is known of the chemistry of the reaction, which according to Lison (1936) was first used in histochemistry by Buffa in 1904. It is given by substances possessing free SH groups such as cysteine and reduced glutathione and has been used for localization of glutathione (Joyet-Lavergne, 1927) and for the free sulphydryl groups of cysteine (Giroud, 1929). These and most other workers have employed frozen sections of fresh tissues in their various researches. Joyet-Lavergne, however, applied the method to the study of alcohol or formalin-fixed sections also, after brief treatment with 2 per cent. trichloroacetic acid to release SH groups from the tissue proteins. Giroud and Bulliard (1932, 1933), suggested that the red colour of a positive reaction might be stabilized by previous treatment of sections with 5 per cent. zinc acetate for a few seconds. Hammett and Chapman (1938), however, experimenting with the nitroprusside reaction on cysteine solutions *in vitro*, found that the presence of zinc acetate decreased the final colour by as much as 75 per cent. These authors evolved a method for application to fresh tissue slices which is given in Appendix 5. In spite of the results obtained by Joyet-Lavergne, I have found that the colour developed by the application of his technique, or the

modification of Hammett and Chapman, to formalin-fixed frozen and paraffin sections and to alcohol-fixed paraffin sections, is invisible under the microscope. The nitroprusside reaction is, in fact, quite inapplicable to fixed tissues. The colour developed when the method is applied to fresh tissue slices, as Hammett and Chapman have stated, does allow a rough estimate of the relative concentration of SH in different regions. Absolute values for free SH in tissue slices are unobtainable.

Phenacyl Methods for SH (Danielli, 1950). These methods depend on the reaction of thiols with reactive bromides or iodides, such as the phenacyl bromides ($C_6H_5COCH_2Br$). The presence of an aldehyde group does not interfere with the reaction. Thus *p*-bromoacetyl benzaldehyde, one of the compounds suggested by Danielli for the purpose, reacts with SH groups in alkaline solution as follows :



The two other compounds, *p*-iodoacetoxybenzaldehyde and *p*-iodoacetamidobenzaldehyde, would react in a similar manner, it was suggested. In each case, after thorough washing of the tissues, the free aldehyde groups of the combined reagent (thioether) should be available for demonstration by means of Schiff's leucofuchsin reagent. Although the aldehyde group of these three compounds might react with other groups in the tissues, such a combination would not subsequently be revealed by means of leucofuchsin. Phenacyl bromides and iodides are not known to react with any tissue groups other than sulphydryls, but the method will not demonstrate all the available SH groups unless the phenacyl halogen reacts more readily with SH than does the aldehyde part of the molecule. It is well known that aldehydes do combine with SH groups. I have no experience of this method in practice, and no technical details have been published. One would expect, from the evidence given by Barron (1951), that a satisfactory histochemical method would not be achieved. This author stated that alkylating agents, of which the above are examples, are the least reactive of the commonly used reagents for thiols. "Reactive halogen groups," he said, ". . . cannot be considered suitable reagents for the detection of SH groups in proteins."

Blocking Methods for SH (Danielli, 1950). The blocking techniques suggested by Danielli for the histochemical demonstration of SH groups are more complicated than those in the preceding section. The five stages of the method, in order, consist of treatment with :

(1) Phenyl mercuric hydroxide, or chloride in alkaline solution, to block SH groups.

(2) Phenyl isocyanate to block NH_2 and aromatic OH groups.
(3) Performic acid to destroy SS groups.
(4) H_2S or dithioglycerol to unblock the original SH groups.
(5) Dinitrofluorobenzene, reduced, diazotized and finally coupled with a suitable phenol.

Explanation of the theory of these successive stages may be necessary.

(1) methyl or phenyl mercury salts react with thiols in the following manner :

(2) The use of isocyanates for the blocking of NH_2 (and tyrosine OH) groups is based on the reaction below. In alkaline solution amino-acids combine with isocyanates to form substituted hydantoic acids.

$$\underset{\text{Amino-acid}}{H_2N.R.COOH} + \underset{\text{Isocyanate}}{R'N:C:O} \xrightarrow{KOH} R'NH.CO.NH.R.COOK + H_2O$$

(3) Toennies and Homiller (1942) showed that performic acid converts cystine into cysteic acid, breaking the SS bonds and yielding sulphonic acids.

$$\begin{matrix} S—CH_2.CH(NH_2).COOH \\ | \\ S—CH_2.CH(NH_2).COOH \end{matrix} \xrightarrow{O} \underset{\text{Cysteic acid}}{HO_3S.CH_2.CH(NH_2).COOH}$$

This step prevents the reduction of SS groups to SH by the reagents employed in the fourth stage.

(4) Reversal of the selective blocking of the SH groups by the phenyl mercury salt is obtained by the provision of other SH-containing compounds, such as H_2S or dithioglycerol.

(5) The use of dinitrofluorobenzene, and its reduction, diazotization and coupling, are described in the section on blocking reactions, below, and in Appendix 5.

This method is complicated and I have not succeeded in obtaining a satisfactory result with either fixed or freeze-dried (unfixed) sections.

Blocking Method for SS Groups (Danielli, 1950). This method, suggested by Danielli for the demonstration of tissue SS groups, has three essential stages and consists in treating sections with the following reagents :

(1) Phenyl *iso*cyanate to block SH groups.
(2) Potassium cyanide to reduce SS to SH.
(3) Dinitrofluorobenzene to combine with SH, followed by reduction, diazotization and coupling.

The method, in my hands, failed to give satisfactory results either with formalin or alcohol-fixed material, or with unfixed freeze-dried tissue sections. Since it is relevant to most of the methods in this section, however, it may be appropriate to consider at this point the suggested reduction of SS to SH with potassium cyanide.

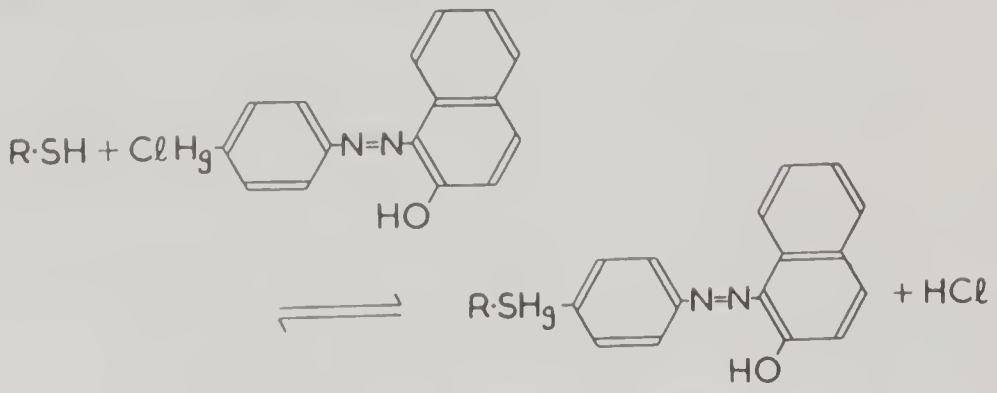
Conversion of SS to SH Groups with KCN. Solutions of potassium cyanide have long been employed in histochemistry for the reduction of tissue SS to SH, prior to demonstration of the latter by the nitroprusside method for instance. Joyet-Lavergne (1927) used 10 per cent. KCN and exposed the tissues for 5 minutes, Lison (1953) recommended 10 per cent. for 5 minutes, and Hammett and Chapman (1938), 5 per cent. for 10 minutes. These last authors, however, stated that SH was destroyed by alkaline solutions and that the alkaline solution of KCN destroyed not only some of the thiol groups initially present in the tissue but also some of those newly converted from disulphide. They observed that cyanide reduction would sometimes establish the presence of SS, but with certainty only when no free SH was present or when the ratio SS to free SH was high. This last condition is present in fixed tissues, but their objection on account of destruction of newly-formed thiol groups remains a valid one.

There are three factors to be considered, the availability of tissue SS groups for reaction with KCN, the speed at which the reaction takes place, and the speed of destruction of thiol groups by the alkaline solution. It is known that the SH groups of free cysteine, and of cysteine combined in various proteins, have different reaetivities with specific SH reagents and that denaturation, on the whole, increases rather than reduces this reactivity. As regards the rate of reaction between SS and KCN, Cuthbertson and Phillips (1945) have shown that 85 per cent. of the disulphide-S of sheep's wool is converted by 1 per cent. KCN in excess, acting for 1 hour at 66°. If the reactivity of SS groups in tissue sections is greater than this, as is most likely, then 10 minutes in 10 per cent. KCN should be sufficiently long to achieve a high degree of conversion and yet short enough to minimize destruction of the newly-formed SH groups by alkali. In praetice, however, although treatment of sections with KCN in the above manner results in an increased number of reactive SH groups as indicated by both Bennett and ferricyanide reactions (see below), many workers have found it far from satisfactory. I now agree with Lillie (1954) that other methods are preferable.

Comparison of KCN with other Reagents. Barrnett and Seligman (1954) tested a number of agents for their efficiency in reducing tissue SS groups to SH ; they were H_2S and its salts, sodium bisulphite, sodium hydrosulphite, sodium thiosulphate, L-ascorbic acid, L-epinephrine, KCN, sodium thioglycollate, cysteine, glutathione,

2-mercaptoethanol, and 2,3-dimercaptopropanol (BAL). Of these they found four particularly useful and efficient ; in descending order these are sodium thioglycollate, ammonium sulphide, KCN and hydrosulphite ($Na_2S_2O_4$). The last was effective only at a concentration of 12 molar and at pH 10·5, while KCN (10 per cent.) required a high pH (11–11·5) and a long period of incubation. Moreover, it was pointed out that this reagent produces only one reactive sulphydryl from each disulphide, together with an inert thiocyanate group. Ammonium sulphide was the most powerful reducing agent and it produced particularly strong staining reactions. However, Barrnett and Seligman showed that part of this staining was an artifact due to the creation of new SH groups by condensation of $(NH_4)_2S$ with tissue carbonyl groups. They recommended that this reagent should be used only with suitable controls (carbonyl blocking reagents ; see Appendix 12, p. 865), especially when exploring new tissues. Sodium thioglycollate was the best and most specific of the four " short-listed " reducing agents. Details of the concentrations used, and of the solvents, etc., will be found in Appendix 5, p. 802.

The Mercury Orange Method for SH (Bennett, 1948, 1951). This method is based on the reaction of mercaptans with organic mercurials like phenyl mercuric chloride, as in the method originally employed by Hellerman *et al.* (1933). These authors also used *p*-chloromercuribenzoate to combine with and block the reactions of SH groups in egg albumin. In an effort to obtain a coloured compound with similar properties to those described above, capable of attachment solely to SH groups, Bennett and Yphantis (1948) synthesized 1-(4-chloromercuriphenylazo)-2-naphthol. This compound is red in colour and it is therefore visible in the tissues if present in sufficient quantity. The reaction with SH groups proceeds as follows :



Bennett (1951), recording the use of his reagent for demonstrating tissue thiols, gave it the name red sulphydryl reagent (RSR)*, and this abbreviation will be used here. Bennett used small pieces of tissue either fixed in 5 per cent. trichloroacetic acid and dehydrated in

* Subsequently renamed mercury orange.

alcohol, or prepared by freeze substitution (Appendix 3, p. 786) from liquid N_2 into *n*-propanol or *n*-butanol at — 20°. In both cases, before staining, the tissue was teased into small fragments. The RSR was employed as a saturated solution in *n*-propanol, toluene, or dimethyl formamide, and staining was usually complete in 2–16 hours. Several variations of the full method are given in Appendix 5.

Using this method Bennett was able to confirm the presence of sulphydryl groups in most of the regions in which they had been detected by means of the nitroprusside reaction. He also located SH in several regions not previously known to contain it, such as in nerve cell bodies and the retinal rods, in axis cylinders and in capillary endothelium.

Specificity of the RSR for Sulphydryl. Aryl or alkyl mercuric halides have been found to react with thiol groups in native and denatured proteins (Anson, 1941 ; Hughes, 1950), and there is good evidence in favour of their absolute specificity for SH. With the RSR, however, there exists the possibility of non-specific attachment to other protein groups in the tissues, or to other non-protein groups. Bennett suggested that four types of binding of RSR might occur in the tissues.

(1) Specific ; by covalent mercaptide linkage to SH.

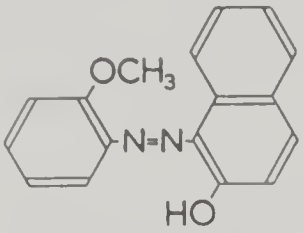
(2) Non-specific ; by hydrogen bonding (Chapter IV, p. 62) to the azo nitrogen or to the oxygen of RSR by suitable H groups in the tissues.

(3) Non-specific ; by van der Waal's forces (Chapter IV, p. 62) between the aromatic rings of RSR and certain tissue components.

(4) Non-specific ; by salt linkages between ionized groups in the tissues (such as phosphate) and the Hg^+ or H^+ of RSR.

The specificity of RSR for SH was established, in the author's view, by the performance of certain critical experiments. It was shown, by substituting phenylazo-2-naphthol for RSR, that the presence of the chloromercury group was essential for the reaction. Using teased muscle fibres Bennett found that no colour was taken up from solutions of phenylazo-2-naphthol, whereas, in solutions of RSR, the muscle fibres were stained red. If the SH groups of the fibres were blocked by alkylation (p. 131) or by mercaptide formation (p. 132), the reaction with RSR was negative, indicating the necessity of the presence of active SH groups. Bennett prepared a number of solutions designed to compete with molecules bound in tissues by the various types of bonding described above and found that only solutions of mercaptans (β-mercaptoethanol, thioglycollic acid, B.A.L., cysteine) would completely reverse the staining of tissues by RSR. Methionine and cystine, which are not mercaptans although they contain sulphur, were quite ineffective.

Bennett's evidence for the specificity of his reaction appears to be fairly strong, and it takes account of most of the possibilities for non-specific attachment to tissue groups. In spite of the results of the author's first critical experiment there remains, however, the possibility of solution in lipid substances to be taken into account. The resemblance of phenylazo-2-naphthol compounds to the Sudan dye Brilliant fat scarlet is very close indeed, as can be seen from a comparison of the formula below with that of RSR.



Brilliant Fat Scarlet

All compounds of this type tend to act as fat stains and RSR is no exception, although it is prevented, by its low solubility in fat solvents, from acting very efficiently in this respect. In formalin-fixed tissues the sites coloured by RSR are mostly also coloured by Sudan black B, though the converse is by no means true. RSR staining, in paraffin-embedded material, is especially strong in red blood cells, and less so in muscle fibres and in the stratum corneum of the skin. In formalin-fixed frozen sections the colour is comparatively less strong in the red cells (Fig. 34). Cannefax and Freedman (1955) tested Nelson's medium, which contains glutathione, cysteine, thioglycollate and other sulphydryl compounds, with RSR and found no visible reaction. By means of *in vitro* tests they established the fact that thioglycollate *prevented* the reaction of RSR with glutathione, cysteine and thiourea. This was suggested as a possible limitation to the use of RSR, but the conditions outlined above will not be operating in the histochemical use of the reagent.

Horowitz and Klotz (1956) used a different azomercurial, 4-(*p*-dimethylaminobenzeneazo) phenylmercuric acetate, in 0·1 M-glycine buffer at pH 9·0, for estimating SH in proteins. They found that the time of equilibration of their reagent with bovine serum albumin (BSA 6×10^{-6}M, Dye 14×10^{7}M, pH 9, 25°) varied between $4\frac{1}{2}$ and 8 hours. With ovalbumin under the same conditions the time varied from $4\frac{1}{2}$ to 17 hours. These results indicate that mercaptide formation is a slow reaction at room temperature, and in using RSR due allowance should be made for this fact. I have tested RSR in glycine buffer, in which it is moderately soluble, as a histochemical reagent, but only diffuse non-specific staining of the tissues is produced. It is not possible to block this with the usual reagents. This emphasizes

the point that different solvents may decrease the specificity of the RSR reagent to a considerable degree.

Mescon and Flesch (1950) used RSR in 80 per cent. ethanol and applied the method to fresh sections of frog tissues prepared by the Adamstone-Taylor technique (see Chapter II, p. 13). These authors attempted to make the procedure quantitative by dissolving SH-bound RSR from the sections by means of amyl acetate, and estimating colorimetrically the amount of dye present in solution. I prefer to use a saturated solution of RSR in dimethylformamide at $\pm$ 40° with thin cold microtome sections. Under these conditions more of the dye is taken up than from alcoholic solutions at room temperature.

Those investigations which I have so far undertaken with RSR agree with those of Bennett in finding the specificity of this compound for SH groups to be high. The original author speaks of the method as "rather insensitive," by which presumably he means that the colour developed is not sufficiently intense for the appreciation of small quantities of thiols. It should be possible to increase the sensitivity of the method by substituting a blue or black chromogenic group in place of the red phenylazo-2-naphthol. Alternatively a method might be evolved using a sensitive thiol reagent such as methylmercury iodide, containing ^{131}I. This would involve demonstration by autoradiography (Chapter XXVII) with its attendant drawbacks.

Mauri, Vaccari and Kaderavek (1954), after considering and testing a number of reactions for thiols, concluded that only the RSR method was sufficiently sensitive and specific. They considered that the insolubility of RSR in aqueous media was responsible for much of the observed difficulty in staining tissues known to contain SH groups. A water-soluble coloured organic mercurial was therefore synthesized, 1-oxy-2-(*p*-chloromercuriphenylazo)-8-aminonaphthalene-3,6-di-sulphonic acid. Blocking and other tests suggested that the specificity of this reagent was as high as that of RSR.

As with all methods which successfully demonstrate SH groups the problem is to determine what proportion of the total available SH is taking part in the reaction. There are two main sources of SH in the tissues, (1) glutathione and (2) protein-bound SH, the latter being either free to react or masked. In Bennett's procedure glutathione is either removed by trichloroacetic acid fixation, or largely preserved by freezing and dehydration in butanol or propanol. Either of these manœuvres will reveal a proportion of the masked SH groups of proteins and enable them to combine with RSR. Those who investigate the functional aspect of sulphydryl histochemistry will wish to show only free SH groups ; investigators of structure desire to show total SH. In practice neither is likely to achieve success, and the final result

of the application of a method such as that described in this section is to demonstrate a number of SH groups which lie between the two extremes. Unless the procedure is very carefully standardized, therefore, comparison between one material and another is likely to be open to criticism.

Details of Bennett's method appear in Appendix 5 and its application to problems of diagnostic pathology is considered in Chapter VII.

The Ferric Ferricyanide Method for SH and SS (Chèvremont and Fréderic, 1943; Adams, 1956). This method, according to the authors, can be performed on sections fixed in formol-saline or in Bouin, provided that the duration of fixation is a few hours only. Frozen or paraffin sections may be employed, preferably the former. It can also be applied to unfixed tissues, in freeze-dried or cold microtome sections, or to paraffin sections after fixation in trichloroacetic acid-alcohol. The method depends on the reduction of a fresh solution of ferricyanide, in acid solution at pH 2·4, by sulphydryl groups in the tissues.

$$2[Fe(CN)_6]^{+++} + 2RSH \rightarrow 2[Fe(CN)_6]^{++++} + RS—SR$$

The resulting ferrocyanide combines with ferric iron (provided as ferric sulphate) to give a precipitate of insoluble Prussian blue.

$$4Fe^{+++} + 3K_4[Fe(CN)_6] \rightarrow Fe_4[Fe(CN)_6]_3 + 12K^+$$

Thus, in principle, the Chèvremont method is the same as Schmorl's method for lipofuscin (see Chapter XXIII, p. 665) and it gives a positive reaction with the latter substance as well as with melanin and argentaffin cell granules.

Distinction between melanin, lipofuscin, argentaffin granules, and protein structures containing SH is not difficult by ordinary histological techniques. Small quantities of lipid reducing substances attached to protein are by no means easy to detect, however. In my opinion the specificity of the Chèvremont method depends on (1) proof of the absence of non-protein reducing groups, and on (2) the specificity of ferricyanide-reduction, as far as protein is concerned, for SH groups alone. Purity of the reagents used is, of course, essential. Considering the second point first : Anson (1939) stated that denatured proteins which contained no cysteine would not reduce ferricyanide. He showed (1941, 1942) that the extent of the reaction, which is catalysed by copper ions and inhibited by cyanide, depended on the concentration of ferricyanide. When twice the stoichiometric amount of the latter was present, 64 per cent. of the SH groups in denatured egg albumin were oxidized, and with fifty times the amount, 94 per cent. Anson found that partial digestion with trichloroacetic acid made the SH groups of denatured protein more reactive ; this recalls Joyet-Lavergne's use of the same reagent. In contradistinction to the above results, which

suggest that the specificity of the method should be high, Mirsky and Anson (1936) stated that some protein groups other than SH were oxidized by ferricyanide, notably tyrosine and tryptophan. Herriott (1947) concluded that, in the absence of evidence to the contrary, it might be presumed that the tyrosine groups in some proteins could be oxidized by ferricyanide, and that ferricyanide-oxidation appeared to be non-specific in some instances and ineffective in others. If this is true, a negative result with the histochemical ferricyanide method cannot be taken to indicate absence of SH.

The histochemical interpretation of the ferric ferricyanide reaction has been thoroughly considered by Adams (1956). He has shown that two pigments are formed when this compound is reduced *in vitro* by a variety of substances. These pigments are Prussian blue and Prussian green. Both are formed during the histochemical performance of the reaction, and the latter forms in small quantities in the medium itself. From this it may possibly be absorbed by tissue components to give rise to non-specific green staining. In any case Adams recommends that the reaction time be limited to 5 minutes and that any green colour be ignored or interpreted with great reserve. This author's theoretical results are also considered in Chapter XXIII, p. 665.

Both tyrosine and tryptophan rapidly produce Prussian blue from ferric ferricyanide *in vitro*, but low cysteine, high tryptophan-containing proteins do not give a positive reaction in tissue sections. Even if the specificity of the method for protein-SH is allowed, however, the objection raised in the first point, above, remains valid. It is possible to distinguish lipids, if present in sufficient quantity, by various techniques (see Chapter XI). It is not possible, by direct histochemical means, to exclude the presence of small amounts of lipid. A different approach to the problem was employed by Yao (1949), who used the Chèvremont method on the eggs of *Drosophila*. He immersed control sections in saturated aqueous mercuric chloride for 1 hour, before treatment with ferricyanide, in order to prevent the reaction of SH groups. Only structures positive in the normally-treated sections and negative in the controls, could then be considered to contain active sulphydryls. A procedure of this kind is essential before the Chèvremont method can properly be used for the demonstration of SH. The mercaptan-forming reagent used by Yao is not the most suitable as the time allowed for the reaction is too short. A saturated solution of phenyl mercuric chloride in 80 per cent. ethanol, or in absolute *n*-butanol, and 48–72 hours exposure, produce a more reliable total blocking of SH groups. Adams (1956) successfully used both types of blocking and also introduced a prior stage of thioglycollate reduction (Appendix 5, p. 802) to convert unreactive SS to reactive SH groups.

In practice the results obtained by the use of the Chèvremont

method resemble closely those obtained with Bennett's RSR. Figs. 34 and 35 illustrate the two methods, applied to frozen sections of skin, and show their essential similarity. For the demonstration of SS groups the Chèvremont method should be employed with a restricted reaction time of 5 minutes, or less, after thioglycollate reduction and with parallel control sections subjected to blocking subsequent to the reduction stage. A blue colour appears in structures containing large amounts of cystine. According to Adams the sensitivity of the method for protein-bound SS is below that of the DDD and alkaline tetrazolium reactions (q.v.). For protein-bound SH the Chèvremont method should also be used with a restricted reaction time, particularly when trichloroacetic acid-fixed sections are employed, and invariably with SH-blocked controls. The sensitivity of the reaction is in this case slightly greater than that of the RSR reaction.

Selective Oxidation Methods for SS (Pearse, 1951; Bonomi, 1951). These methods, of which technical details appear in Appendix 5. are based on the selective oxidation of cystine by performic acid. Toennies and Homiller (1942) showed that performic acid reacts only with tryptophan, cystine and methionine among the amino-acids, whereas, according to Nicolet and Shinn (1939) periodic acid attacks tryptophan, methionine, cystine and the α-hydroxy amino-acid serine. In earlier work I found that periodic acid, together with $KMnO_4$, H_2O_2 and perchloric acid, were unsuitable. Only performic and peracetic acids were satisfactory in practice. Theoretical considerations indicated that a major portion of the reaction product from performic acid oxidation of cystine was likely to be cysteic acid (alanine-β-sulphonic acid), and the histochemical problem was resolved into the demonstration of this substance in the tissues. A method for the identification of sulphonic acids *in vitro* was suggested by Latimer and Best (1937), which involved the formation of phenylhydrazides, but the colour of these was too faint to be visible in tissue sections. An aryl hydrazide, 2-hydroxy-3-naphthoic acid hydrazide (NAH) was therefore used instead. Final coupling with a diazonium salt was necessary to develop the full colour of the reaction. Using performic oxidation, followed by NAH and diazotized *o*-dianisidine, a positive result was obtained in structures containing keratin.

Since NAH had been usefully employed as an aldehyde reagent (Pearse, 1951), Schiff's solution was automatically suggested as an alternative and from this the performic acid-Schiff procedure was worked out. Alternative methods used to demonstrate the reaction product were staining in dilute aqueous solutions of methylene blue and combination with cobalt salts, subsequently revealed by conversion to black cobalt sulphide. Examples of the application of the three methods are shown in Figs. 36, 37 and 38. Oxidation of tissues with

performic acid was shown to give rise to histochemically detectable reaction products particularly in two classes of material. These were keratin, with which we are here concerned, and unsaturated lipids of the phosphatide class. The application of the performic and peracetic acid-Schiff techniques (PFAS, PAAS) to lipids is considered in Chapter XI, p. 325.

Subsequent Development of the PFA Reactions for Cystine

As I remarked in the first edition of this work, the PFAS method is still largely of academic rather than of practical interest. It has given rise, however, to a large amount of experimental work undertaken with the idea of explaining the mechanism of the reaction in the case of keratin. This work, and my own which preceded it, is given below in a separate section. First, it is necessary to describe the development of the reaction into one of considerable practical importance.

Of the two alternative reagents originally used in place of Schiff's reagent to demonstrate the sulphonic acid reaction product of performic acid oxidation of cystine-containing structures, methylene blue (M/2000, pH 2·6) was regarded as the most satisfactory and specific. Lillie, Bangle and Fisher (1954) used Azure A and thionin as alternatives, following oxidation by performic and peracetic acids and by bromine in CCl_4. All these variants produced metachromatic staining of keratin but of no other tissue component, so that the methods are inadmissible as reactions for cystine-containing structures in general. A different approach was made by Scott and Clayton (1953). These authors used Lugol's iodine, periodic acid and acidified permanganate followed by Schiff's reagent or by Gomori's (1950) aldehyde fuchsin. A positive result with Schiff's reagent was obtained only after permanganate oxidation, but with aldehyde fuchsin all three types of oxidation were sufficient. Unfortunately, as pointed out by Bangle (1956) and by Braun-Falco (1956), though the sensitivity of aldehyde fuchsin is high, its specificity is low since it combines with aldehydes by non-polar addition as well as with acid radicals by salt linkage.

There is little doubt that performic acid is the most suitable reagent, on both chemical and histochemical grounds, for the oxidation of cystine, and Adams and Sloper (1955, 1956) have combined it with an acid solution of the phthalocyanin dye Alcian blue (Chapter IX, p. 259) for their studies on the distribution of the neurosecretory substance (Fig. 39). *The performic acid-Alcian blue method* has a high specificity but a relatively low sensitivity, and it therefore demonstrates only high concentrations of cystine in the tissues. Notwithstanding, there are two important uses of the method in applied histochemistry. These are the demonstration of NSS in various species and the division of

the anterior pituitary mucoid (basophil) cells into cystine-rich and cystine-poor groups. Both these applications are further considered in Chapter VII, pp. 178 and 179.

As alternatives to performic and peracetic acids it is possible to use permolybdic and pertungstic acids (Earland *et al.*, 1955), and these can be used histochemically. In the case of permolybdic acid some molybdenum remains in combination with the oxidized keratin, and this suggests a possible development of the method.

The Mechanism of the Performic acid-Schiff Reaction

In working out the mechanism of the reaction with Schiff's solution in the case of performic acid-oxidized keratin, it was established that traces of performic acid, or of the formic acid and H_2O_2 from which it was made, could not be responsible for the development of a red colour. It was further shown that a sulphonic acid (*p*-toluene sulphonic acid) produced no colour when added to Schiff's solution *in vitro*. The corresponding sulphinic acid (*p*-toluenesulphinic acid), however, produced a strong but slowly developing reaction under the same circumstances. This finding suggested that the oxidation of cystine in tissue sections produced not only alanine-β-sulphonic acid but also, perhaps as an intermediate stage, alanine-β-sulphinic acid ($HO_2S.CH_2.CH(NH_2)CO_2H$). According to Cecil (1950) the most stable of the intermediate reaction products of cysteine and cystine is the sulphinic acid. *In vitro*, however, oxidation of cystine to cysteic acid was observed to take place without giving a Schiff-positive reaction product.

Experimental evidence (Pearse, 1951) suggested that in performic acid-oxidized sections three possible groups could account for the appearances observed when these were subsequently treated with Schiff's solution, methylene blue, or cobalt salts. The three groups are sulphonic (SO_3H), sulphinic (SO_2H), and aldehyde. In order to establish responsibility more accurately the reactions concerned were carried out *in vitro* and by a modified Coujard technique (Coujard, 1943). In this method the various reagents are dissolved or suspended in a serum-gelatin mixture which is used to draw lines or bands on the surface of a glass slide. The slides are subsequently dried and exposed to a fixative. They are well washed in running water before use in histochemical reactions. The results of these manœuvres appear in Table 8 (on page 113).

It seemed clear that both sulphonic and sulphinic acids might be responsible for the reaction with methylene blue and possibly with Schiff's reagent also, since in this case some colour was produced by sulphonic acid in the Coujard technique. Aldehydes are not responsible for the results with methylene blue but might be responsible for some of

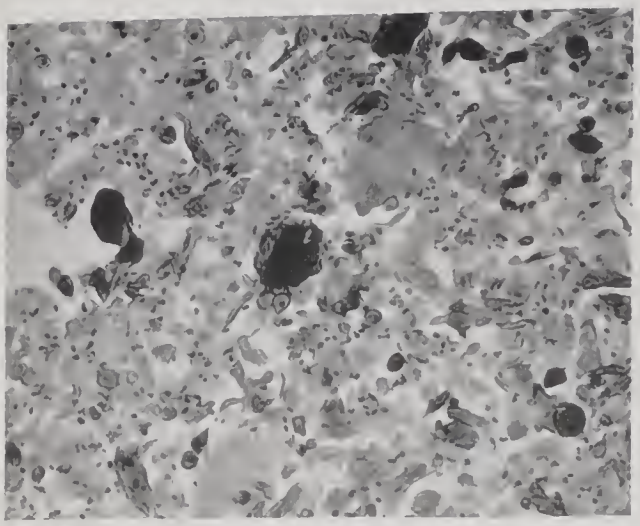


FIG. 38. Keratin masses in a squamous-cell carcinoma, metastasis in a lymph node. Performic acid, cobalt nitrate/sulphide, carmalum. × 105.

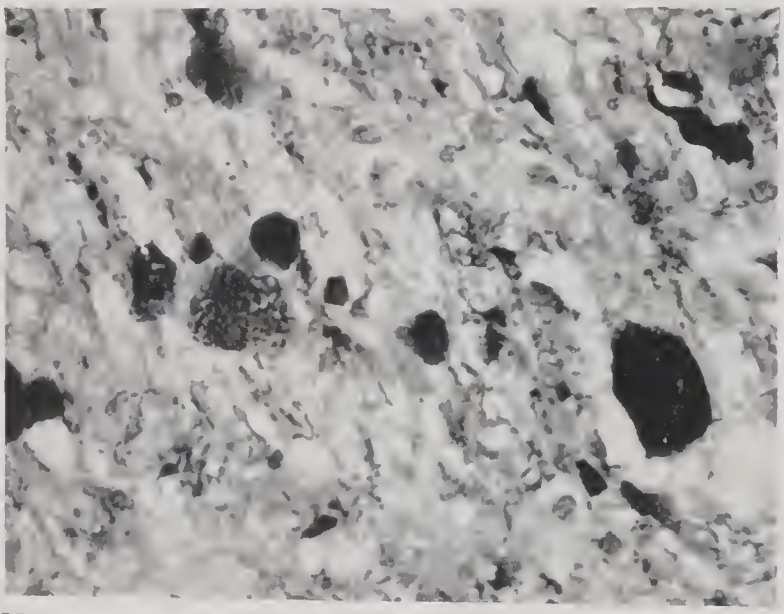


FIG. 39. Human neurohypophysis. Shows large and small masses of neurosecretory substance. Performic acid—Alcian blue. × 510.

FIG. 40. Rat skin. Positive reaction in hair shaft and epidermal keratin layers. Alkaline tetrazolium reaction. × 130.

[*To face p.* 112.

FIG. 41. Rat skin. Positive reaction in hair shaft (blue) and in other structures (reddish purple). DDD reaction. × 130.

FIG. 42. Human liver, as Figs. 30 and 31. Paraprotein crystals. DDD reaction. × 210.

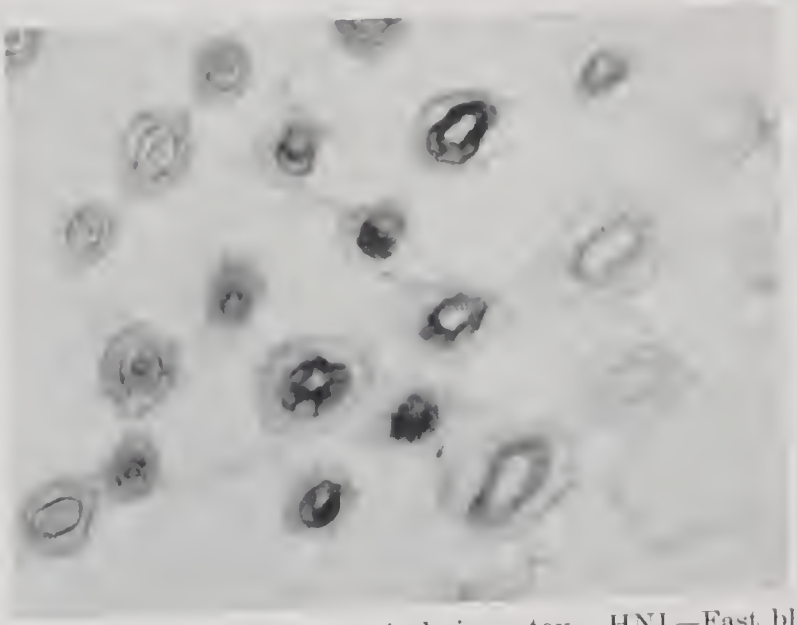


FIG. 43. Rat skin. Strong reaction in hair cortex. HNI—Fast blue B salt. × 210.

TABLE 8

Reactions of Sulphonic, Sulphinic, and Aldehyde Groups

	In vitro		Coujard			
Radicle	Schiff	Ammoniacal silver	Schiff	NAH-dianisidine	Ammoniacal silver	Meth. blue pH 2·6
SO_3H *p*-toluenesulphonic . .	−	−	faint +	+	−	+
SO_2H *p*-toluenesulphinic . .	+	−	+	faint +	−	+
CHO phthalaldehyde . . .	+	+	+	+	+	−

the colour both with Schiff and the NAH-dianisidine reagent. Provided that a low pH was employed the methylene blue technique was found to be more specific than the other two methods for the demonstration of sulphur-containing reaction products. At higher pH levels methylene blue reacts with other groups, such as COOH, produced in the tissues by oxidation.

Lillie and Bangle (1954) disagreed with the above hypothesis and suggested that the PAAS (and PFAS) reactions of hair cortex were due to an unsaturated complex of probable lipid nature. They showed that while iodine could oxidize sulphinic acids *in vitro*, it could not prevent subsequent development of the Schiff reaction in oxidized hair. This finding, with which I concur, certainly suggests that the sulphinic acid hypothesis, as it stands, is untrue. The alternative hypothesis of a highly polymerized, partially oxidized lipid cannot be dismissed on account of the fact that a PFAS or PAAS reaction is obtainable after fixation in boiling $CHCl_3/CH_3OH$ since, as Lillie has pointed out, these reactions are positive in the case of ceroid (Chapter XXIII, p. 666) after similar treatment.

Findlay (1955a) found that α- and β-keratin membranes prepared from human hair by the method of Alexander and Earland (1950), which involves treatment with peracetic acid in the initial stages, were strongly Schiff-positive without further oxidation. He also found that alternative aldehyde reactions gave negative results with these membranes and with oxidized hair. In the case of the latter the Ashbel-Seligman reagent (Chapter XII, p. 338) gave a red colour rather than the blue which is normally produced by tissue aldehydes. Findlay also found that it was impossible to block the positive Schiff reaction with aldehyde blocking reagents such as cyanide, phenylhydrazine, hydroxylamine and aniline. He considered that the Schiff reaction of peracetic acid-oxidized keratin is probably non-aldehydic and that a relatively intact keratin structure is required for its production. No alternative mechanism for the reaction was forthcoming.

Fractions of isolated wool oxidized with peracetic acid were studied by Earland and Knight (1955). Their two fractions, designated α- and β-keratose, correspond to Findlay's α- and β-keratins. Neither fraction would form an ammonium salt, although cysteic acid does so readily *in vitro*, and this failure was explained by Alexander, Fox and Hudson (1951) as being due to the formation of sulphonamides rather than sulphonic acids. Weston (1955), however, found no SO_2NH in the infra-red absorption spectra of the two fractions, but SO_3H was definitely present. Earland and Knight suggested, therefore, that strong salt links were formed between the sulphonic acid groups and basic residues ($^+NH_3$) in the tissues and that these prevented the normal reactions of a sulphonic acid from taking place. While these findings do not provide a direct explanation for the PAAS reaction of keratin they suggest the direction in which further inquiries should proceed.

The investigations of Landing and Hall (1956a) add nothing to the solution of the PAAS problem as far as keratin is concerned and this remains, for the present, in equipoise.

Tetrazolium Methods. Barrnett and Seligman (1952) first suggested the use of ditetrazolium chloride (BT) for the demonstration of SH and SS groups in the tissues. In later papers (1953 and 1954) further details of its application were given, and in the final method celloidin-covered sections were incubated for 8–12 hours in a mixture containing alkali and KCN as well as BT. Because of its higher redox potential (greater ease of reduction) Rogers (1953) preferred to use 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) for the demonstration of SH in frozen sections of skin. He incubated these at pH 7·4 for up to 2 hours and produced a red colour in components containing SH. These conditions are not adequate for the demonstration of total reactive SH groups, and INT forms large crystals, especially in sections which have been stored.

I used three different salts in my early studies of the so-called alkaline tetrazolium reaction (Pearse, 1953, 1954) ; these were neotetrazolium (NT), BT, and 2,5-diphenyl-3-(4-styrylphenyl)-tetrazolium chloride (M & B 1767). All three were used in 0·1 M-glycine buffers at between pH 11·9 and 12·8 and at 60°. The results with BT were clearly superior to those of the other two salts in that the staining which resulted was non-particulate. Components in the tissues giving rise to a positive alkaline tetrazolium reaction were divided into three groups :

(1) Cystine or cysteine-containing.
(2) Lipid or lipofuscin.
(3) Reducing sugar-containing.

An additional miscellaneous group included such entities as enterochromaffin cell granules and adrenochrome.

Barrnett and Seligman (1954) suggested that reductants such as sugars, adrenalin, and lipid aldehydes would fail to react because they would not be present in paraffin sections. This is not quite true. Protein-bound sugars are retained in any case, and lipid aldehydes also. Adrenalin is preserved, as adrenochrome, by oxidant fixatives, and this substance gives a positive reaction. When the reaction is carried out at pH 12 and at 60° such components undoubtedly react ; at 37° the effect of alkaline hydrolysis upon the sugar-containing components is usually insufficient to allow them to stain. Lowering the pH of the reaction to 11·0 has a similar effect, and sugar-containing components largely fail to react. Lipid aldehydes, however, still do so.

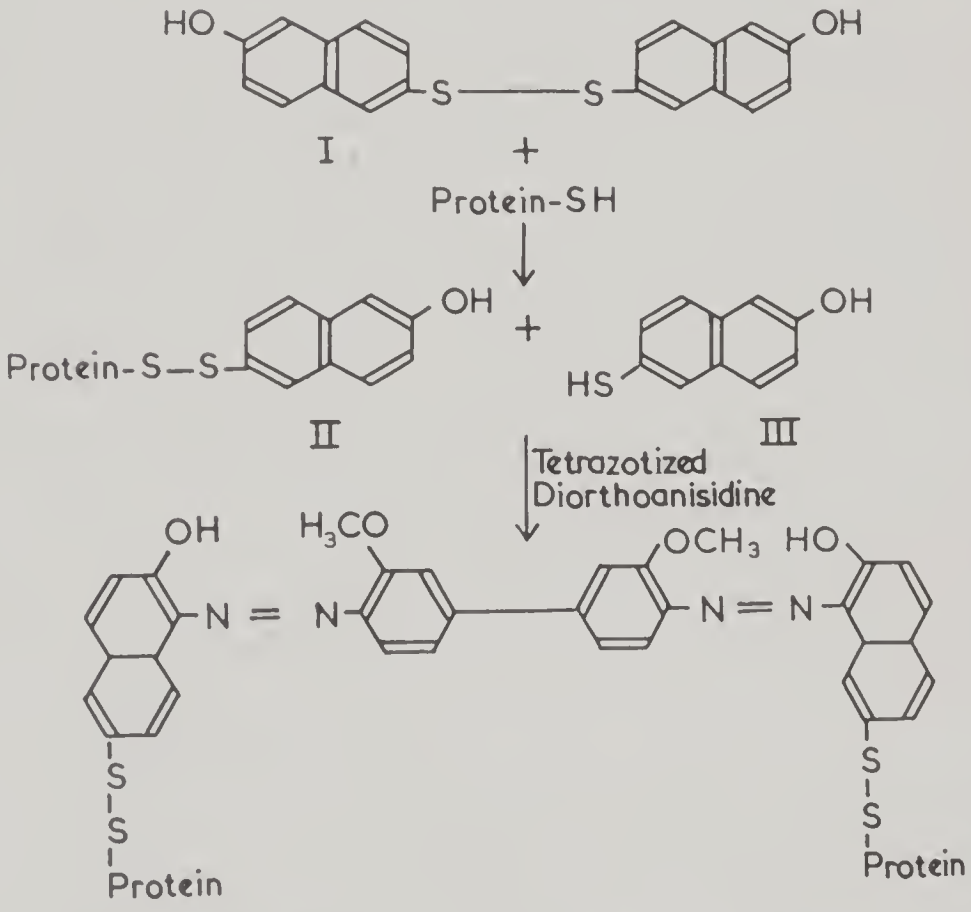
Both SS and SH groups have been considered to take part in the reaction. Barrnett and Seligman (1954) believed that under the conditions they employed (pH 11, 0·37°, KCN) the reaction of sulphur containing tissues was due to SH groups alone. These were (1) original SH groups present in the tissue, and (2) alkali cyanide-reduced SS groups. According to Cuthbertson and Phillips (1945) at 60° and pH 12 cystine breaks down to form combined lanthionine, combined aminoacrylic acid, H_2S and free sulphur. Beaven and Holliday (1952), on the other hand, consider that SH and SOH groups are formed as intermediate reaction products and that SOH is immediately converted into S or H_2S. At the pH employed in the alkaline tetrazolium reaction (Pearse, 1953) both SH and H_2S, and probably also free sulphur, should reduce tetrazolium salts effectively, and the reaction therefore shows tissue disulphides and sulphydryls without distinction. Findlay (1955b) made a series of experiments to test the validity of the alkaline tetrazolium reaction for tissue sulphydryls. He found that the relative concentration of the reactants made a great difference to the rate and efficiency of the reaction *in vitro*. When the tetrazolium salt was present in excess cysteine produced relatively little reduction between pH 10·8 and 12·6. At this last level, however, provided that the concentration of cysteine was low, the reaction became 100 per cent. efficient. Findlay also compared the DDD and tetrazolium reactions and found a number of inconsistencies. Some of these have not been explained, but his interpretation of the tetrazolium reaction of the nucleoli as due to SS groups is open to the alternative explanation that it is due to reduction by the ribose sugar of RNA. Reversibility by ribonuclease is a point in favour of this second hypothesis.

The low specificity of the alkaline tetrazolium method renders it relatively unsuitable as a method for SS and SH. If it is required for the demonstration of these groups alone then the conditions specified by Barrnett and Seligman should be employed. Their technique is

given in Appendix 5, p. 807, and the results are illustrated by Fig. 40, p. 112. Since the sensitivity of this method, as opposed to its specificity, is fairly high it may be useful as a control technique for alternative methods.

The Dihydroxy-Dinaphthyl-Disulphide (DDD) Method for SH and SS

This method (Barrnett and Seligman, 1952, 1954 ; Barrnett, 1953) is based on the use of a reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulphide, which was synthesized especially for the purpose. The disulphide grouping is the only specific oxidizing agent for sulphydryls and the reagent thus has absolute specificity as far as the first part of the histochemical reaction is concerned. The full mechanism of the reaction is shown in the following series of equations :



In the first stage the reagent (I) combines with protein-SH by splitting to form one protein-naphthyl disulphide (II) and one free naphthyl mercaptan (III). The latter, and any excess of the original reagent adsorbed on the tissues, is removed by washing in a series of alcohols and finally in absolute ether. For the final development of colour the sections are treated with a diazonium salt which combines with the naphthol moiety to form an azo dye. For this purpose the original authors used Fast blue B. The results of the method are shown in Fig. 41, and also in Fig. 42, p. 113.

The theoretical specificity of the DDD reaction for SH has been confirmed by a rigorous series of control procedures. No colour is produced in the tissues after oxidation of SH groups with iodine (p. 132) or after blocking with specific reagents such as iodoacetate (p. 131) or N-ethyl maleimide (p. 131). Partial failure to block the reaction, often observed when mercurials such as phenyl mercuric nitrate are used, can be attributed entirely to the fact that such blocking is easily reversible. Furthermore, the DDD reaction can be prevented by interposing, before the final stage of colour development, a stage of treatment with excess sulphydryl (glutathione).

Bahr (1957) carried out a series of model experiments to test the specificity of the DDD reaction in which he used the stable diazotate of 4-amino-3,6-dimethyl-4-nitroazobenzene (Fast black salt K) in place of Fast blue B. An alternative which can be used with satisfactory results for the same purpose is the diazotate of 4-benzoylamino-2,5-dimethoxyaniline (Fast blue RR). By means of a back titration with DDD and ovalbumin Bahr showed that there was a linear relationship between the amount of colour developed in the reaction and the number of protein-bound SH groups available. He considered that the advantage of using Fast black salt K was not confined to the greater amount of colour which developed, but also the absence of a two-coloured result such as occurs when a bis-diazonium salt such as Fast blue B is used.

According to the original authors of the method a blue colour (di-coupling) signifies a high concentration of tissue SH groups, whereas a red or pink colour (mono-coupling) indicates a sparse distribution of reactive groups. Intermediate colours are commonly observed. Only the di-coupling reaction is shown in the final equation for the DDD method (p. 116). A pink blush in connective tissues, which are supposedly sulphydryl free, has been observed by many workers. Such non-specific colouring may result from two causes : (1) a diazonium reaction with histidine and tyrosine residues ; and (2) inefficient extraction of unused DDD and of the free reaction product 6-thio-2-naphthol. The second of these is undoubtedly the most important and it is probably responsible for the staining of elastic tissue by the DDD method. Elastica has an affinity for phenols and naphthols which has not been satisfactorily explained but which is not easily reversible by simple extraction with solvents.

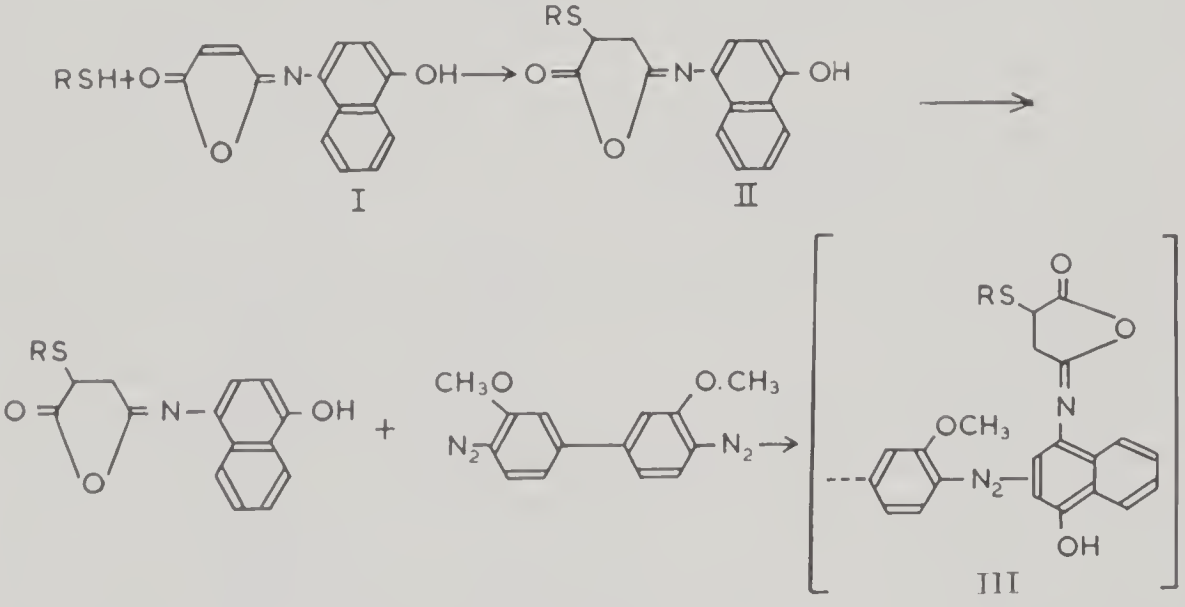
Reference has also been made to the lack of contrast obtained with the DDD method. This is largely inevitable with a method of high sensitivity capable of revealing small concentrations of SH in the tissues. By comparison a method of low sensitivity such as Adams' performic acid-Alcian blue (p. 111) produces a sharply contrasting picture which is satisfying to the æsthetic sense of the histologist. The

histochemist brought up in this discipline must unlearn some of his acquired discriminatory sense.

I consider that, of all the methods for SS and SH so far discussed in this chapter, the DDD method must form the base line for any investigations in applied histochemistry. I employ it in parallel with the RSR method, using fresh frozen sections in this case in spite of the theoretical difficulty of comparing frozen with paraffin sections. The application of SS and SH methods to practical problems is further considered in Chapter VII, p. 175.

The α-Naphthyl Maleimide and other Naphtholic Reactions for SH

Seligman, Tsou and Barnett (1954) introduced a further series of naphtholic reagents for the demonstration of protein-bound SH groups. Following the use of N-substituted maleimides as specific blocking reactions for SH these authors tested a number of hydroxynaphthyl-N-maleimides as histochemical reagents and found the 1-4 derivative N-(4-hydroxy-1-naphthyl) *iso*maleimide (HNI) to be the best. Trichloroacetic alcohol-fixed sections were reacted with the maleimide at pH 7·5 for 30 minutes, and after extraction of excess reagent the final colour was developed with Fast blue B.



Later the same authors (Barrnett, Tsou and Seligman, 1955) described the synthesis and application of other naphtholic reagents for SH, including 4-hydroxy-1-naphthyl mercuric acetate and 4-(*n*-iodoacetyl)-amino-1-naphthol. Reaction with these two compounds was stated to be slower than with DDD or HNI. Price and Campbell (1957) have shown that the sensitivity of HNI for thiols is high and that the reaction proceeds rapidly *in vitro*. In paper chromatograms as little as 0·3 mμ-mole could be detected. These authors also found that HNI made

by the original method of Tsou, Barrnett and Seligman (1955) contained at least two components and that in some cases it was necessary to separate them chromatographically and to use only the faster component. This does not appear to be necessary in histochemical practice.

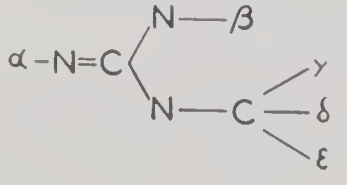
The reaction is particularly dependent on pH and should be performed at pH 8·0 or higher, in contradistinction to the blocking reaction with N-ethyl maleimide (p. 131), which can be carried out best under acid conditions. A control section treated only with Fast blue B salt is necessary in the case of any tissues containing phenolic substances. This refers particularly to plant tissues.

Of the three reagents mentioned above I have used only HNI. This reacts readily with tissue SH groups and the ultimate picture closely resembles that produced by the DDD method. Apparent differences constantly noted with certain tissue components are presumably due to stereochemical factors. The application of the method to rat skin is shown in Fig. 43, p. 113. Price and Campbell also found that the final reaction product (III) could be demonstrated on paper chromatograms by means of its intense red fluorescence (366 mμ). This observation can form the basis of a histochemical U.V. method since, fortunately, the reaction product in the tissues gives the same red fluorescence. The resulting increase in sensitivity may make this method the method of choice when tissues containing few reactive SH groups are being investigated.

Details of the reaction, incorporating modifications introduced by Price and Campbell, are given in Appendix 5, p. 808.

Reactions for Arginine

The colour reaction for arginine originated by Sakaguchi (1925) is highly specific but the development and preservation of the colour are technically difficult. The reaction was first modified for use in histochemistry by Baker (1944), later by Serra (1944) and, independently, by Thomas (1946). According to the second author, it was to be regarded as specific for guanidine derivatives in which a hydrogen atom of one amino group was substituted by an alkyl or acyl radical. Thomas, however, stated that guanidine derivatives in which a hydrogen atom of one or both amino groups was substituted would react but that those in which both hydrogen atoms of a single amino group were substituted did not do so. Baker (1947) determined that a positive Sakaguchi reaction occurred with compounds having the general structure



where α and $\beta = H$ or CH_3. Such substances include the naturally occurring amino-acids galegine, agmatine and arginine. In practice, a positive reaction is not given by any amino-acids occurring in human tissues other than arginine, nor is it given by guanidine, creatinine or urea.

In the Sakaguchi reaction a red colour is produced when arginine reacts with α-naphthol and hypochlorite or hypobromite in alkaline solution. Hypobromite is more specific than hypochlorite according to Macpherson (1942), who stated that excess hypobromite quickly destroyed the colour already produced. In performing the reaction it is usual to add urea with the α-naphthol to take up the excess hypobromite when this is subsequently added. Macpherson pointed out that this manœuvre was only partly effective because although the formation of the coloured compound is instantaneous its destruction by hypobromite begins immediately. Unless the proportion of urea and hypobromite is identical, and unless mixing is efficient, the best results will not be obtained. The original reaction of Thomas, which employed hypochlorite, was performed at room temperature instead of at 0°–4° as in Serra's and the original Sakaguchi method, and this is undoubtedly an advantage where no cold room is available. If one follows Macpherson's view, Serra's is the method of choice because it uses the more selective reagent (hypobromite) and because it avoids the long period in α-naphthol-hypochlorite which characterizes the original Thomas modification. Baker's (1947) modification employs hypochlorite which he prefers to hypobromite for three reasons. First, the validity of the reaction is based on Sakaguchi's work in which he used hypochlorite ; secondly, the latter is more easily available ; and thirdly, rapid development of colour is not necessary in histochemistry. More recently Thomas (1950) has introduced further refinements into the method which include the provision of tetraethylammonium hydroxide as a stabilizer for the colour developed.

With Serra's method the maximum colour is satisfactory, but this is developed while the section is still immersed in the staining solution after addition of the final portion of hypobromite. Usually, however, before the section can be removed and mounted for inspection considerable loss has occurred. Serra's " stabilization " of the colour, by repeated changes of glycerol and mounting in this medium, takes place too late to prevent this earlier loss of colour, due to excessive action of hypobromite. As Macpherson observes, there is an optimum amount of hypobromite (or hypochlorite), and it is the difficulty of finding this optimum which provides the main problem in performing the arginine reaction on tissue sections.

I have used Serra's method, the original and the newer Thomas' methods, and Baker's method. Of these, the simplest and most

convenient is the last. It gives excellent and fairly stable results on tissues fixed in a variety of formalin-containing or alcoholic fluids, and also on freeze-dried material. The application of Baker's method is illustrated in Fig. 44, p. 128. Thomas' (1950) method was a great improvement on his original version, but complicated to use. It offers a more permanent preparation than Baker's pyridine-chloroform. Warren and McManus (1951) described the use of 8-hydroxyquinoline (attributed to Sakaguchi) in place of α-naphthol in the histochemical method for arginine. They considered that the stability of the colour developed was greater than with the older methods, but Gilboe and Williams (1956) could find little difference between α-naphthol and oxine in a series of tests.

The oxine reagent of Sakaguchi was also used by Carver, Brown and Thomas (1953) as the basis of an arginine reaction permitting final mounting in a synthetic medium (Permount) with considerable stability of colour. This method is also given in detail in Appendix 5, p. 800. If permanence is regarded as unimportant I still recommend the use of Baker's variant of the α-naphthol-hypochlorite procedure as preferable to the others, either in its original form or using 2,4-dichloro-1-naphthol (McLeish, Bell, La Cour and Chayen, 1957) in place of α-naphthol. This compound gives a strong reddish-orange colour which is sufficiently stable, in an alkaline medium, to allow photometric measurements to be made.

Serra (1946) recommended the arginine reaction as a means of distinguishing basic proteins in the tissues, a distinction which can be achieved by simpler and conventional methods of staining. Nevertheless, in spite of practical drawbacks, when applied to tissue proteins demonstrated by other methods as basic, it affords a means of distinction between arginine-rich histones and arginine-poor globins. This distinction can also be made histochemically by the use of specific methods for tryptophan, since this amino-acid is present in traces, if at all, in histones.

Blocking and Conversion Reactions for Protein End-Groups

A large number of such reactions have been employed or suggested for use in protein histochemistry. Many of them are not specific for a single group but react in varying degrees with others. The reactions are considered below in groups corresponding to those in which the chromogenic end-group reactions have already been considered. In Appendix 5 the methods of application are listed without reference to their specific use but approximately in the order in which they are considered here.

Reactions for Amines (Schiff's base formation, deamination, oxidative deamination, dinitrophenylation, acetylation, benzoylation,

nitrobenzoylation, phosphorylation, aryl sulphonation, substituted urea and thiourea formation).

Schiff's Base Formation. The condensation of an aldehyde with a primary amine or amino compound takes place readily, with the formation of a weakly basic compound called variously a Schiff's base, an anil, or an azomethine.

$$R.NH_2 + OCH.C_6H_5 \longrightarrow R.N : CH.C_6H_5 + H_2O$$

The main example of Schiff's base formation used as a blocking reaction is formalin fixation, and it is thus absolutely necessary to avoid this procedure when total NH_2 groups are to be demonstrated. That the reaction with formaldehyde is incomplete is shown by the positive reaction for NH_2 obtainable by most of the specific chromogenic methods after formalin fixation.

Deamination. Nitrous acid reacts with all types of amino groups in the tissue proteins but most rapidly with the α-amino groups.

$$R.NH_2 + HONO \longrightarrow R.OH + N_2 + H_2O$$

According to Springall (1954), if one uses the Van Slyke (1929) reagent, the α-amino nitrogen is evolved in 4 minutes, the ϵ-amino nitrogen from lysine in 30 minutes, and the guanidino-nitrogen of arginine in 6 hours. In histochemical practice nitrous acid mixtures are usually used for 1–12 hours at room temperature and, according to Lillie (1954), the acetic acid mixtures are more rapid in action than those based on HCl, in spite of their higher pH levels.

Oxidative Deamination. It is possible to carry out this reaction in a number of ways. It forms the first stage of the ninhydrin and alloxan-Schiff methods (p. 87) and is, in fact, seldom used purely as a blocking reaction.

$$R.CHNH_2.COOH + \tfrac{1}{2}\,O_2 \longrightarrow R.CO.COOH + NH_3$$

Monné and Slauterback (1951) used both ninhydrin and chloramine-T for blocking by oxidative deamination, and Burstone (1955) showed that the latter would produce Schiff-reactive aldehydes in tissue proteins.

Dinitrophenylation. This reaction was introduced into protein chemistry by Sanger (1945), and it depends on the reaction of 2,4-dinitrofluorobenzene (DNFB), under mild alkaline conditions, with terminal α-amino groups and with the ϵ-amino groups of lysine.

$$R.NH_2 + F.C_6H_3(NO_2)_2 \longrightarrow RHN.C_6H_3(NO_2)_2 + HF$$

Dinitrophenyl amino-acids are bright yellow, but the colour is not sufficient for histochemical purposes. In practice the method is not used for blocking amino groups, or for their demonstration following

addition procedures which are described in the section below on tryptophan, tyrosine and histidine.

Acetylation, Benzoylation, Nitrobenzoylation. These are variants of the same essential process, the Schotten-Baumann reaction, by means of which acyl derivatives of primary and secondary amines and amino compounds are produced. These reactions are not used as blocking methods for NH_2 groups, but *p*-nitrobenzoyl chloride and dinitrobenzoyl chloride have been used by Burstone (1955) for demonstration of acylated groups in tissue proteins after procedures similar to those described below for DNFB.

Phosphorylation. This procedure was introduced into histochemical practice by Landing and Hall (1956a), who suggested that some of the phosphoryl groups introduced into tissue proteins were attributable to esterification of the amino groups. What proportion of the available NH_2 groups is affected by phosphorylation is unknown.

Acyl Sulphonation. This process has been used in protein chemistry for the identification of terminal amino-acid residues. Christensen (1945) used *p*-toluene sulphonyl (tosyl) chloride, and Udenfriend and Velick (1951) employed *p*-iodophenyl sulphonyl (pipsyl) chloride for the same purpose. Under mild alkaline conditions a sulphonamide is formed with free α-amino groups :

$$R.NH_2 + I.C_6H_4.SO_2Cl \longrightarrow R.NH.SO_2C_6H_4I + HCl.$$

Aryl sulphonation has not been used as a blocking reaction, but might well be used as a sensitive method for demonstrating protein-bound amino groups by substituting the fluorescent compound dimethylaminonaphthalene sulphonyl chloride (see Chapter VI, p. 140) for the above reagents.

Substituted Urea Formation. Primary and secondary amines and amino compounds react with aryl *iso*cyanates at room temperature to form substituted ureas. Other compounds such as water, alcohols and phenols, which contain a mobile hydrogen, also react and must be excluded. The process has been used to give fluorescent carbamidoproteins for use in the Coons technique (Chapter VI, p. 144), but not for blocking.

Reactions for Carboxyl Groups (Methylation, Hydroxyalkylation)

Methylation. This process can be carried out, in chemical practice, in a number of different ways. Compounds used for the purpose include dimethyl sulphate, methyl iodide, methyl bromide and diazomethane. Fraenkel-Conrat and Olcott (1945) described a method for methylating proteins using methanol-HCl mixtures, and this procedure was successfully adopted for histochemical use by Wigglesworth (1952), and later by Fisher and Lillie (1954). While the majority of methylating

agents affect other groups, particularly NH_2 and tyrosine-OH, Fraenkel-Conrat and Olcott considered that the methanol method specifically blocked only carboxyl groups :

$$R.COOH + CH_3OH \overset{HCl}{\rightleftharpoons} R.COO.CH_3 + H_2O.$$

This reaction is reversible *in vitro* by treatment with HCl, but in the tissues this result can only be achieved with strong oxidizing agents such as $KMnO_4$.

Fisher and Lillie used the method in a study of its effects on tissue metachromasia and basophilia. They found that methylation quickly reversed the metachromasia of most tissue components, and even the basophilia of the nucleic acids was not resistant. They concluded that SO_3H and PO_4 groups were also blocked by the process, which could not therefore be regarded histochemically as specific for COOH. Furthermore, after more drastic methylation with methanol-HCl the PAS reaction of mucins was considerably reduced. It appears, therefore, as if 1,2-glycol groups may also be affected.

Diazomethane was employed by Peters (1955) in his studies on the mechanism of silver staining. Blocking of this was obtained with diazomethane, but owing to the unspecific nature of the reaction this could not be interpreted as indicating that COOH groups are responsible for silver staining. Bello (1956) has suggested that specific esterification of carboxyls can be obtained with thionyl chloride in methanol, but this method does not appear to have been tested histochemically.

Hydroxyalkylation with 1,2-epioxides was suggested by Fraenkel-Conrat (1944) as a method for blocking protein-bound COOH groups, but its specificity is not high, since at various pH levels NH_2, tyrosine-OH and SH groups also react.

Reactions for Tryptophan, Tyrosine and Histidine (Oxidation, Acylation, Benzoylation, Dinitrophenylation). Danielli (1947, 1950) put forward a series of reactions to be used with the coupled tetrazonium reaction in order to distinguish each of the above-mentioned amino-acids. Of these reactions benzoylation and dinitrophenylation have already been mentioned as possible blocking reagents for amines which are not much used for this purpose in histochemistry. The third reagent, performic acid, is used here as a specific oxidizing agent for tryptophan.

The application of all three reagents to the demonstration of individual aromatic amino-acids is described in the following section in the way in which it was set forth by the original author, although in the light of modern protein chemistry the interpretation of the results obtained has undergone revaluation. Table 9, taken from Danielli (1947, 1950), amended, shows the use of the three blocking and

eliminating reagents in conjunction with the coupled tetrazonium reaction.

Differential reagents of the types suggested by Danielli can be used in three ways :

(1) To prevent, by chemical combination, the subsequent staining of a given group. The presence of this group is recognized by deficiency in staining by comparison with unblocked sections.

(2) To convert or destroy the selected group. The use of the word " blocking " is not strictly permissible in this case and the reaction, unlike many of those in the first category, is always irreversible.

(3) To combine with a given group in the tissues, leaving part of its own molecule reactive. The latter is then made visible by some additional process.

The principle of the first type of reaction is shown by the use of DNFB to block tyrosine. This amino-acid fails to react by the coupled tetrazonium method after treatment with ice-cold saturated DNFB in alkaline alcohol. Reduction in the colour obtained, by comparison with control sections, was originally thought to be due to tyrosine. A glance at Table 9, however, shows that cysteine and lysine are also concerned. Used in this way the reaction might be useful as a control method for the Millon reaction (*q.v.*), but it is seldom used for the purpose.

TABLE 9

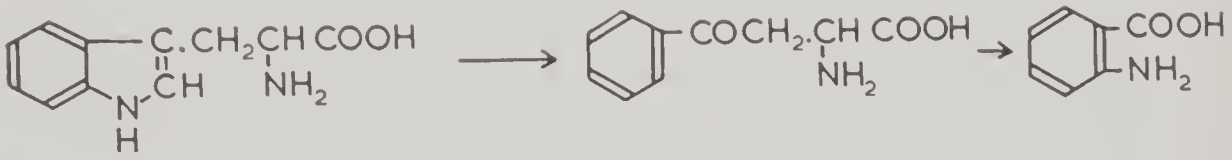
The Coupled Tetrazonium Reaction. (Blocking and Eliminating Reagents)

Substance	Alone	After di-nitro fluorobenzene	After performic acid	After benzoyl chloride
Tyrosine	+	−	+	−
Tryptophan	+	+	−	−
Histidine	+	−	+	+
Lysine	+	−	+	−
Cysteine	+	−	+	−
Arginine	+	+	+	−

Performic Acid. The principle of the second type of differential method can be illustrated by the use of selective oxidation with performic acid. It was shown by Toennies (Toennies and Homiller, 1942 ; Toennies, 1942 ; Bennett and Toennies, 1942), that, of the commonly occurring amino-acids only cystine, methionine and tryptophan are oxidized by performic acid. After treatment with this acid, therefore, the indole ring of tryptophan fails to react by the

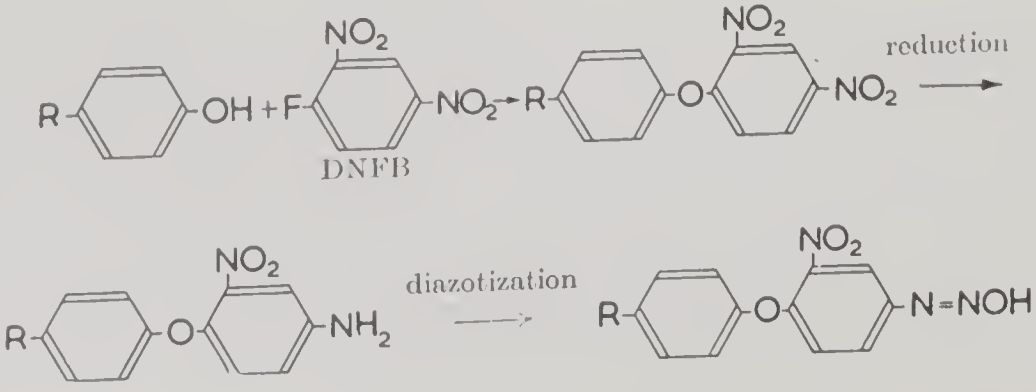
coupled tetrazonium method while tyrosine and histidine continue to do so. The presence of tryptophan is inferred from the reduction in colour caused by pre-treatment with performic acid. This compound is produced by mixing hydrogen peroxide and formic acid in suitable proportions (see Appendix 5) and it is used at room temperature. Danielli found that performic acid had a strong solvent action on chromosomes and this effect he eliminated by buffering to pH 4·5.

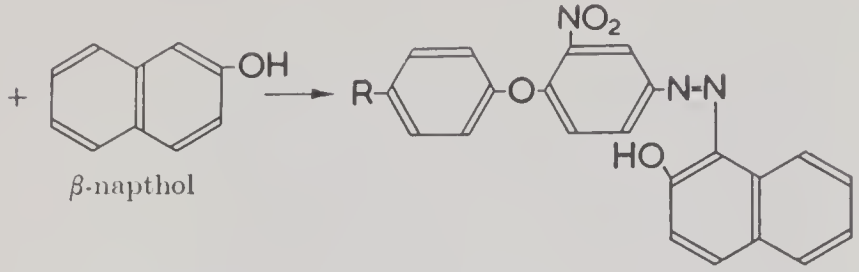
Persulphate. It has been shown by Boyland, Sims and Williams (1956) that alkaline persulphate can break the pyrrole ring of tryptophan at the 2,3-bond to give a number of acid-labile products :



Using a 2 per cent. solution of $K_2S_2O_8$ in 0·5 N-KOH, I have found that 16 hours' incubation at 22° is sufficient to reverse the various specific methods for tryptophan discussed earlier in this chapter. The nature of the reaction product in the tissues has not been elucidated.

Dinitrofluorobenzene. The use of the third type of differential method of Danielli is illustrated by the reaction between the aromatic hydroxyl group of tyrosine and dinitrofluorobenzene (DNFB). The compound formed by the reaction of these two substances is colourless. However, if the 4-nitro group of the attached DNFB is reduced to NH_2, then diazotized with nitrous acid at 0° and finally coupled in cold alkaline solution with a suitable phenol or aromatic amine, the location of the tyrosine-DNFB compound becomes strongly coloured. Danielli used 1-amino-8-naphthol-3 : 6-disulphonic acid (" H-acid ") as his final coupling agent and I have also found it suitable. This reagent gives a rich reddish-purple colour which is insoluble in alcohol or xylene ; sections stained with it can, therefore, be dehydrated and mounted in the ordinary manner. The chain of reactions involved in this second type of blocking is thought to proceed as follows :





Unfortunately, the use of this method for demonstrating tyrosine is complicated by the fact that DNFB reacts with other groups in the tissues such as SH groups, the free α-amino groups of proteins and, to some extent, with the ϵ-amino groups of lysine (Sanger, 1945, 1950). According to Porter (1950) the imidiazole group of histidine also reacts to give a colourless product with DNFB. Danielli overcomes these problems by using specific agents to block SH and NH_2 groups before treatment with DNFB. Such agents are naphthoquinone, which blocks both groups, or nitrous acid followed by iodoacetamide. After treatment with the above compounds the aromatic OH of tyrosine is still free to react with DNFB. Danielli does not make it clear whether the imidiazole group of histidine is also blocked by the NH_2 and SH reagents mentioned. If it were not, the DNFB schedule for tyrosine (Appendix 5, p. 796) would indicate histidine in addition.

The original method, in my hands, was quite unsatisfactory when performed upon conventionally fixed tissue sections, although a colour was developed with freeze-dried tissues. Burstone (1955), however, showed that this failure was due to the use of weak reducing agents. The substitution of sodium hydrosulphite for stannous chloride results in the strong staining of a number of tissue components such as collagen and elastic tissue. The reaction is notably diminished by deamination, and more so by acetylation. Burstone suggests that the DNFB-H-acid sequence is relatively specific for hydroxyl and primary NH_2 groups and that it can reasonably be employed as such. His method appears in Appendix 5, p. 796.

Benzoylation. The final process indicated in Table 9, namely benzoylation, must now be explained. It belongs to the first group of " blocking " reactions, and is carried out by treating sections with 10 per cent. benzoyl chloride in dry pyridine for 8–16 or more hours at room temperature. Acyl halides (carboxylic and sulphonic acid halides) are employed by chemists for the characterization of amines and amino compounds and common examples in the three groups are acetyl chloride, benzoyl chloride and *p*-toluene sulphonyl chloride. These halides form derivatives with amino groups,

$$NH_2.R.COOH + C_6H_5CO.Cl \rightarrow C_6H_5CO.NH.R.COOH + HCl$$

alcohols and phenols,

$$R.OH + C_6H_5CO.Cl \rightarrow C_6H_5CO.OR + HCl$$

and they therefore block the reaction of the last group with diazonium and tetrazonium salts. The chemist arranges for the hydrochloric acid evolved to be taken up by performing these reactions in strongly alkaline solutions and this method was used by Mitchell (1942) in an earlier histochemical adaptation. Danielli (1947), however, used a 10 per cent. solution of benzoyl chloride in dry pyridine which acted as vehicle and catalyst, absorbing the hydrochloric acid produced by the reaction but disturbing the tissues far less than the strong alkali of the earlier method. Acetyl and *p*-toluene sulphonyl chlorides require high temperatures, if rapid results are required, and the reactions must therefore be conducted in pyridine at 100° under a reflux condenser. In practice, acetic anhydride is usually used in place of acetyl chloride.

$$NH_2.R.COOH + (CH_3CO)_2O \rightarrow$$
$$CH_3CO.NH.R.COOH + CH_3COOH$$

Tissues firmly attached to slides stand up to such treatment remarkably well but benzoyl chloride in pyridine, since it can be used at room temperature, is clearly the reagent of choice.

As can be seen from Table 9, treatment with benzoyl chloride should prevent the subsequent reaction of lysine, cysteine, arginine, tyrosine, and tryptophan by the coupled tetrazonium method. Danielli (1947) suggested that the last traces of histidine might be difficult to eliminate by benzoylation and later (see Chapter VIII, p. 191), he showed that in tissue sections histidine was in fact resistant although it is easily benzoylated *in vitro*. In my experience the hæmoglobin of the red cells, which contains about 8 per cent. of histidine by comparison with 3·5 per cent. in serum albumin, stains very moderately by the tetrazonium reaction even without previous benzoylation, and not at all after it. Evidently, when combined in hæmoglobin, the imidiazole group of histidine is not entirely free to react with tetrazonium salts. According to Mitchell (1942) and Danielli (1947, 1950), the positive tetrazonium reaction of benzoylated tissues was due to purine and pyrimidine-containing compounds. These compounds were considered to be entirely responsible for the positive reaction of the nuclei and both authors used the full method as specific for nucleic acids. Danielli, at that time, believed that the purines and pyrimidines were stable to benzoylation, but he did observe that the chromatin of the nuclei stained less intensely after this process. He attributed this reduction in staining to the reversal of that portion of the original staining due to the presence of amino-acids.

Using samples of DNA and DNA-protein, I found that the purines and pyrimidines of DNA would not react *in vitro* by the tetrazonium reaction (see Chapter VIII, p. 190). A survey of the histochemical

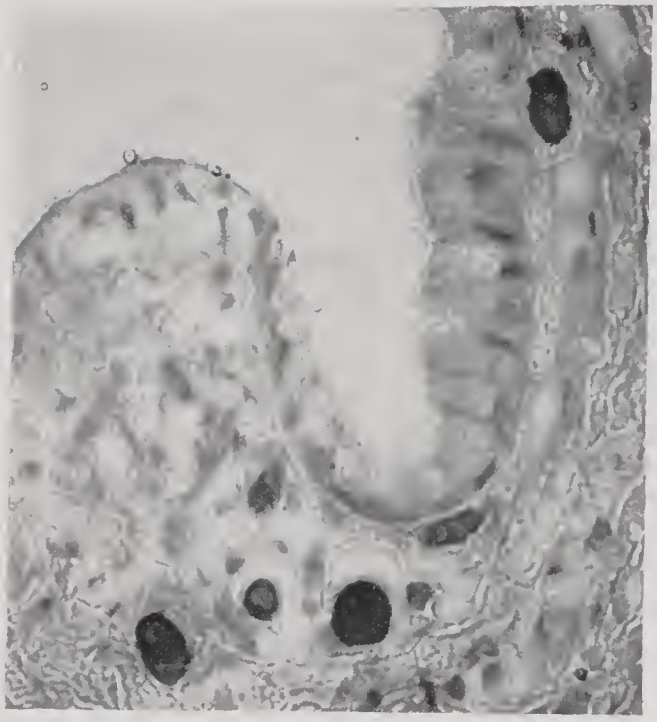


FIG. 44. Human cervix uteri. Simple protein crystalloid bodies containing large amounts of arginine. Sakaguchi reaction (Baker modification). × 400.

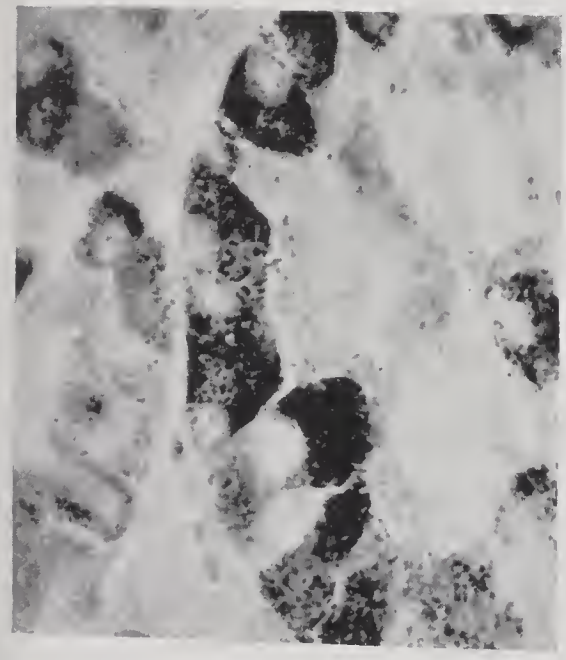


FIG. 45. Human anterior hypophysis. Specific staining of the β-granules after the application of moderate heat followed by 18 hours' benzoylation. Coupled tetrazonium reaction. × 550.

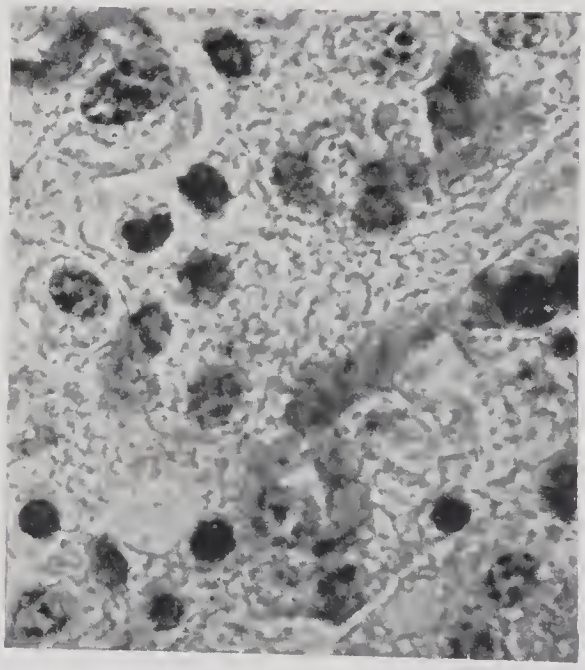


FIG. 46. Plasma cells, and at middle right-hand edge, a group of simple protein Russell bodies. Fourteen hours' benzoylation at 22°. Coupled tetrazonium reaction. × 700.

[*To face p.* 128.

effects of the reaction, in various tissues, was therefore undertaken in the hope that it might prove possible to attach to it a definite chemical or physical significance.

When paraffin sections of tissues fixed in formalin-containing or alcoholic fixatives, or freeze-dried tissues fixed in absolute alcohol, were used, a large number of structures were found to react by the coupled tetrazonium method after as much as 12–18 hours benzoylation. As expected, nuclear chromatin, nucleoli and some cytoplasmic ribonucleoprotein continued to react, but, in addition, collagen and reticulin fibres invariably stained as strongly as in the control sections not exposed to benzoylation. This effect was found to be partly due to the action of even mild heat, such as that involved in drying the sections after mounting on slides in the usual manner (see Chapter VII, p. 159). The pyridine, used as solvent for benzoyl chloride, was found to have no effect on the tetrazonium reaction on its own account. Other structures which continued to stain only after being subjected to mild degrees of heating were fibrin and fibrinoid deposits, the β granules of the anterior pituitary gland (Fig. 45), intracellular gastric, intestinal (Plate Ic), and tracheal mucin, the mucoid granules of the salivary glands, Paneth cell granules, mast-cell granules, Russell bodies (Fig. 46), the ground substance of cartilage and the intercellular substance of arterial walls. If benzoylation was continued for a period longer than 16 hours all the above structures and even the nucleoproteins gradually ceased to react. Alternative treatment with acetic anhydride (10 per cent. in pyridine at 100° for 8 hours) rendered all these structures negative with the exception of nuclei, nucleoli, collagen, reticulin and some cytoplasmic ribonucleoprotein. Even brief acetylation sufficed to render the pituitary β granules negative.

Incubation with ribonuclease for only a short period prevented the subsequent reaction of the nucleoli and cytoplasmic ribonucleoprotein ; but, on the other hand, after performing a Schneider extraction with hot trichloroacetic acid (see Chapter VIII, p. 214, and Appendix 8) so that all Feulgen-positive material was removed from the nuclei, it was still possible to demonstrate the nuclear chromatin by means of the tetrazonium reaction both before and after benzoylation. This observation clearly indicates that the reaction of nuclear chromatin is not due to its purine and pyrimidine content. No single relevant chemical characteristic, other than the possession of some or all of the six reactive amino-acids, is known to be shared by all the materials listed above, although the majority of them are either acid mucopolysaccharides or mucoproteins. It was therefore considered that the resistance of these amino-acids, and particularly of the aromatic ones, to benzoylation was due to physical causes, inherent in the spatial arrangement of the polypeptide chains of the proteins containing them,

making them susceptible to some kind of rearrangement through mild heating. It is significant that the mucin of the brush border of the duodenum and free mucin in the stomach, which may be presumed to have been acted upon by enzymes, are not stained by the coupled tetrazonium reaction after benzoylation, although they are still brilliantly stained by the periodic acid-Schiff reaction for mucopolysaccharides (Chapter IX, p. 231). Plates Ib and Ic illustrate this particular point.

Thus the conclusion to be drawn from the results of the tetrazonium reaction after benzoylation, though the reaction is primarily dependent on the presence of the three aromatic amino-acids, is in fact entirely a physical one. The differences in resistance to benzoylation and to acetylation, on the part of various materials, may be explained as a function of the smaller molecule of acetic anhydride and of the high temperature at which it is used. Benzoylation at 100° reverses the tetrazonium reaction of all structures nearly as quickly as does acetylation at that temperature. Benzoylation must always be carried out with a fixed schedule, the time of exposure being based on the examination of a series of sections fixed by whatever method is to be employed in subsequent investigations. Sections which are destined to be used for comparison should preferably be carried through the entire routine together. This process includes any heating, even at low temperatures, which the materials may have to undergo at any stage in their preparation. It is probable that when tissues fixed in a variety of fixatives have been sufficiently studied by the above techniques, the variable performance of some of the structures mentioned will be found to be explicable on a physical basis. The application of the coupled tetrazonium reaction is further considered in Chapters VII and VIII.

Nitrobenzoylation. Burstone (1955) adapted the Schotten-Baumann blocking procedure by substituting *p*-nitrobenzoyl chloride for the unsubstituted acid chloride and using the Danielli sequence (reduction, diazotization and coupling) to render visible the sites of reaction in the tissues. An intense reddish-purple colour is produced in collagen and elastic tissue and a less strong colour in mucins and in the nuclei. No blockage is caused by deamination but, as might be expected, benzoylation reverses the reaction entirely. Erythrocytes do not stain by this method and the results, in general, are difficult to interpret. The reaction is worthy of further study, however, and an elucidation of the mechanism by which gastric parietal and chief cells are distinguished in the dog and guinea-pig, but not in other species, might well be illuminating.

Iodination. Richards and Speakman (1953) investigated the iodination of tyrosine side chains in keratin under various conditions. They

found that with 0·1 N-I_2 in aqueous solutions only 75 per cent. of the available reactive groups were iodinated (64 hours, 22°, pH 9). When, on the other hand, they used 0·78 N-I_2 in ethanol for 72 hours at 25° they were able to produce 100 per cent. iodination. Histidine was affected to a negligible degree and tryptophan and proline not at all. Gemmill (1956) studied the iodination of phenolic compounds related to thyroxine, using aqueous solutions of iodine in KI at pH 12·05 (0·05 N-KOH). By varying the concentration of iodine Gemmill found that only at higher concentrations did di-iodination take place.

Landing and Hall (1956c) made use of the fact that iodination (0·3 per cent. I_2 in 0·6 per cent. aqueous KI) at pH 10 for 24 hours will block the subsequent reaction of tyrosine with Millon's reagent. They considered that tryptophan would be blocked by this procedure and that any positive coupled tetrazonium reaction obtained after iodination would be due to histidine. Unfortunately for the specificity of this reaction there is strong evidence that complete iodination of tryptophan does not take place and, in fact, it is possible to obtain a positive Adams' reaction for tryptophan after iodination. Even if this were not so the reaction of other amino acids (see above) would have to be taken into account. According to Lillie (1957b) histidine is also iodinated, but he notes that, according to Ramachandra (1956), the reaction is much slower than with tyrosine.

Reactions for SH and SS (Carboxyalkylation, Maleimide block, Mercaptide block, SH-oxidation, SS-reduction)

Carboxyalkylation. Rapkine (1933) described the formula of alkyl carboxy derivatives of thiols by treatment with halogenated fatty acids at alkaline pH levels :

$$Pr—SH + I—CH_2COO \rightarrow Pr—S—CH_2COO + HI$$

The reaction has been observed to occur also with iodoacetamide, iodo-ethyl alcohol and with brominated fatty acids. Iodoacetamide is more reactive than iodoacetate, though the latter is more frequently used in protein and enzyme histochemistry. All existing histochemical SH reactions are blocked irreversibly by prior treatment with iodoacetate.

Maleimide Block. N-ethyl maleimide reacts with tissue SH groups as shown in the equation for α-naphthyl *iso*-maleimide on p. 118. Shects, Hamilton, De Gowin and King (1956) have drawn attention to the failure of N-ethyl maleimide to react with SH groups in intact erythrocytes. They conclude, from their experimental data, that inability to penetrate the red cell envelope is responsible for this. Apart from this circumstance tissue SH groups are entirely prevented

from reacting in histochemical tests by prior treatment with N-ethyl malcimide.

Mercaptide Block. A large number of compounds containing the mercurie ion have been employed as blocking reagents for SH groups. Among these are $HgCl_2$, phenyl mercurie acetate, methyl mercury iodide, *p*-chloromercuribenzoic acid and *p*-chloromercuriphenyl sulphonic acid. The reaction on which SH blocking by organic mercurials is based is given on p. 104.

In all cases, as pointed out by Horowitz and Klotz (1956), the reaction for SH with mercury compounds is slow, many hours being required, at pH 9·0 and 25°, for equilibration to occur. Apart from this drawback, which makes it difficult to be certain that all reactive SH groups have been blocked, the mercaptide linkage is not stable and it may be broken by competition with other SH reagents. The most active of these, such as DDD, cannot usually be successfully blocked by organic mercurials.

SH-oxidation Reactions. Tissue sulphydryls can be reversibly oxidized to disulphides by means of iodine. For this purpose Barrnett and Seligman (1954) used I_2 in KI buffered to pH 3·2 with 0·01 N-HCl. It has been suggested that at this pH level only SH groups are oxidized. Bennett (1951) used 0·001 M-I_2 in *n*-propanol, which was just as effective though probably less specific in action. Subsequent reduction with any of the reagents described below restores the original SH groups.

As an alternative to I_2 Bennett used a mixture of H_2O_2 and $FeCl_3$, in water or in *n*-propanol, but this has no advantages over the two iodine solutions mentioned above.

SS-reduction Reactions. A large number of reagents has been employed for reducing SS to SH groups. The strongest of these, namely zinc-HCl, titanous chloride and sodium amalgam are not used in histochemistry. They bring about the reaction

$$R.S—S.R \rightarrow R.SH + HS.R$$

That is to say, one cystine molecule gives rise to two cysteine molecules. Cyanides have been employed both by chemists and histochemists for the same purpose, but here the reaction is as follows (Clarke, 1932) :

$$R.S—S.R \rightarrow R.SH + CN.SR$$

where only one cysteine group per cystine residue is produced. Another reagent in common use for the same purpose is sodium sulphite. According to Cecil and McPhee (1955) the reaction in this case proceeds in the following manner :

$$R.S—S.R. + SO_3^{--} \rightarrow R.S^- + R.S.SO_3^-$$

and

$$R.S^- + H^+ \rightleftharpoons R.SH.$$

In this case also, therefore, only one cysteine group is produced.

Barrnett and Seligman (1954) tested sodium bisulphite ($NaHSO_3$),

sodium hydrosulphite ($Na_2S_2O_4$), sodium thiosulphate ($Na_2S_2O_3$), ascorbic acid, L-epinephrine, potassium cyanide, sodium thioglycollate, cysteine, glutathione, 2-mercaptoethanol and 2,3-dimercaptopropanol at various pH levels from 8 to 11·5 and at various temperatures from 37° to 50°. They found, as stated earlier in this chapter, that the most effective reagents were thioglycollate, $(NH_4)_2S$, KCN and $Na_2S_2O_4$. Of these the first is now usually employed in histochemical practice as the standard reagent for the purpose.

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CHAPTER VI

FLUORESCENT ANTIBODY METHODS
(IMMUNOHISTOLOGY)

Introduction. In the first edition of this book (1953) a small subsection of the chapter on proteins and amino-acids dealt with the localization of proteins by the fluorescent antibody method of Coons (Coons, Creech and Jones, 1941 ; Coons, Creech, Jones and Berliner, 1942). Since this time the method has been increasingly employed as a research tool and the results obtained by various workers are sufficiently important to justify treatment of the method and its applications in a separate chapter.

Principle of the Method

The essentially simple principle of the fluorescent antibody technique is perhaps best illustrated, in the first instance, by describing the steps through which it was developed. Basically it depends on the fact that dye molecules can be attached to a protein without impairment of its function as an antibody. This was first shown by Reiner (1930), who found that the agglutinating titre of pneumococcal antisera was unchanged after coupling with diazotized atoxyl. He suggested that sera labelled in this way might be useful for studying quantitative aspects of the antigen-antibody reaction. A coloured antigen was produced by Heidelberger, Kendall and Soo Hoo (1933), who coupled R-salt-azo-benzidine to ovalbumin, and a year later Marrack (1934) produced colour-labelled antibodies (anti-typhoid and anti-cholera), in the same manner, which he showed would react with the homologous organisms and colour them pink. Coons, in unpublished studies, repeated this work with rabbit anti-pneumococcus serum, but found that although the organisms were agglutinated the colour they developed was less than adequate. He therefore tested the fluorescent labelling method originated by Creech and Jones (1940, 1941). These authors prepared the *iso*cyanates of several aromatic hydrocarbons and coupled them to various proteins to give a highly fluorescent product. They analysed a fluorescent β-anthryl carbamido derivative of rabbit anti-pneumococcus III serum and found that it contained two hydrocarbon groups per molecule of protein. This was sufficient to give an intense (blue) fluorescence even in dilute solution. Their product agglutinated Type III pneumococci only and the clumped organisms showed macroscopic and microscopic fluorescence.

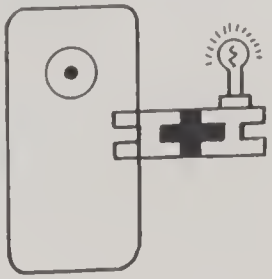
It was soon found, however, that the blue fluorescence of the β-anthryl carbamido-protein could be distinguished only with difficulty from the normal blue autofluorescence of the tissues. In order to overcome this difficulty and to provide fluorescence in a contrasting colour, fluorescein-4-*iso*cyanate was prepared and coupled to the antibody. The product showed the high intensity greenish-yellow fluorescence of fluorescein in high dilution. The essential feature of the fluorescent antibody method, thus developed, is the precipitation of labelled antibody from a concentrated solution placed on a tissue section containing the antigen in reactive form, that is to say substantially undenatured in most cases. Conditions must be such that the labelled antibody becomes firmly attached only to sites containing reactive antigen. The excess of labelled antibody is washed away before examining the section with the fluorescence microscope.

The method of Coons and his collaborators has opened up an entirely new field of protein histochemistry by allowing the histochemist to make use of the very high specificity of immune reactions in the localization of antigenic proteins.

Multiple Layer Techniques

The original method, depending on the attachment of ambient antibody to fixed location antigen was illustrated graphically by Mellors (1955) in a convenient manner which is reproduced below.

With this method a large amount of work has already been carried out, but certain deficiencies are evident. For instance it is incapable of localizing tissue antibodies. To serve this and other purposes, multiple-layer techniques have been evolved. The simplest of these makes use of the interposition of a layer of antigen between the tissue antibody (fixed location) and the same antibody coupled *in vitro* with fluorescein *iso*cyanate.

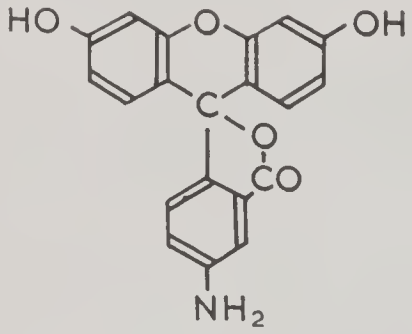


An alternative application of the layer technique has been to use fluorescent anti-globulin for the detection of layered specific antibody attached to the antigen in or on the cell. This technique differs from the other in the fact that the intermediate layer is acting as an antibody on the one hand but as an antigen on the other.

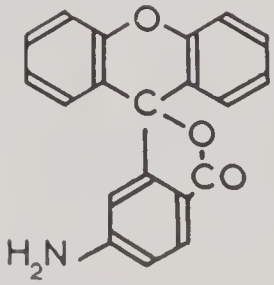
This fluorescent anti-globulin has been useful especially in cases where the antigen is difficult to obtain and the production of the specific fluorescent antibody thus difficult to achieve.

Practice of the Method

Preparation of Fluorescein *Iso*cyanate. Fluorescein-4-*iso*cyanate is prepared by passing phosgene into a solution of the amine in acetone. The amine, which occurs in two isomeric forms (I and II) can be



I



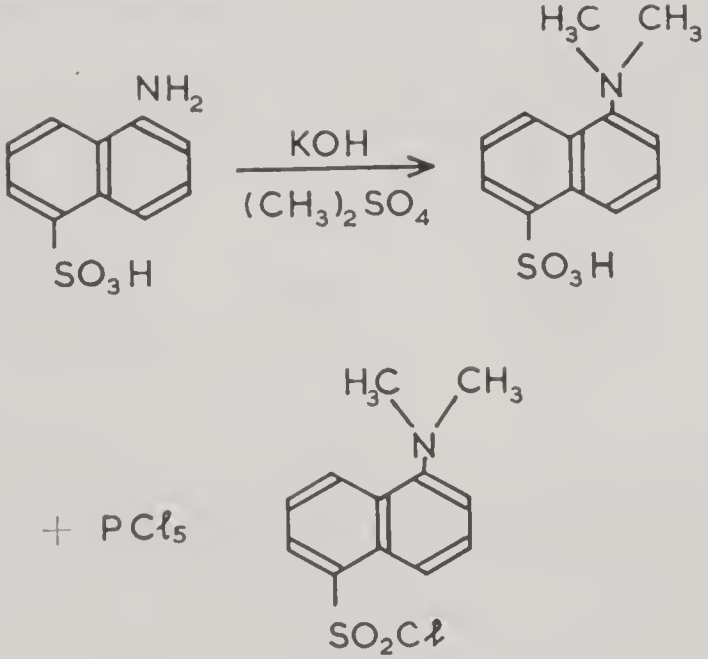
II

synthesized by the method of Coons, Creech, Jones and Berliner (1942) according to the details given by Coons and Kaplan (1950), which are also given in Appendix 6, p. 810. It is available commercially in the United States but only occasionally, at the present time, in this country. Improvements in the methods of synthesis have been introduced and separation of the two isomers has been carried out by de Repentigny and James (1954) using chromatography with a kieselguhr column. The stability of the two isomers is markedly different. Isomer I is completely stable for a long period, but Isomer II breaks down within a short time and is thus useless for keeping as a stock preparation pending the final synthesis of the *iso*cyanate.

Although Marshall (1951) reported that sealed ampoules of the

acetone solution of the *iso*cyanate would remain stable for a year in the dark the majority of workers find a much lower stability. A period of 8 weeks was suggested by Coons (1956) as the minimum period of satisfactory storage. This is to be regarded as the period for which storage is definitely acceptable. Probably the maximum permissible length of storage does not exceed 3 months as a rule. It is worthy of note that Moulton (1956) found that his conjugates (hyper-immune canine distemper serum) had a higher antibody titre when prepared from fresh *iso*cyanate than from the older, stored, product.

Alternative Fluorescent Labels. The technical difficulties attached to the preparation and purification of fluorescein amines, and objections to phosgenation as the final stage of preparation, have naturally resulted in a search for alternative methods of linking a fluorescent label to proteins. Only two compounds have been seriously suggested as alternatives to fluorescein *iso*cyanate. The first of these, 1-dimethyl-aminonaphthalene-5-sulphonyl chloride (DANSYL) was used by Weber (1952) and Laurence (1952) in their studies on polarization of fluorescence. This compound is easily prepared by methylation of 1-aminonaphthalene-5-sulphonic acid (DANS) and subsequent treatment with PCl_5 (Appendix 6, p. 812).



It couples with protein α-amino groups by covalent bonding to form sulphonamides. Between one and three groups per molecule of protein can be attached in this way and the resulting conjugate gives an intense yellow fluorescence.

Clayton (1954) used this method for labelling antisera prepared against certain embryonic antigens, but, in my original studies (with H. Mayersbach) carried out in 1956, it compared unfavourably with fluorescein *iso*cyanate in two respects. First, a substantial amount of

the compound was adsorbed on to the protein which had been labelled by covalent bonding, and prolonged dialysis was necessary before the dialysate ceased to be fluorescent. Secondly, when this point was reached the resulting conjugate was considerably less fluorescent than the fluorescein conjugate prepared from the same protein.

In further extensive studies carried out independently, however, Mayersbach (1958) has found that the difficulty of successful dialysation after DANSYL conjugation is confined to whole serum or to albumen fractions. Only short dialysis is necessary with conjugated globulins and the final fluorescence compares favourably with that given by fluorescein *iso*cyanate conjugates, except that the wavelength of the light emitted is closer to that of normal tissue autofluorescence. If whole serum or albumin conjugates are to be prepared a very large volume of dialysate is essential. The fact that conjugation can be carried out with concentrations of acetone as low as 10 per cent. constitutes an advantage which DANSYL possesses over fluorescein *iso*cyanate in that much less denaturation of the protein occurs during conjugation.

Hartley and Massey (1956) investigated the reaction of 1-dimethylaminonaphthalene-5-sulphonic acid with various amino-acids. They found : (1) a very stable bonding with α-amino groups, resistant to both acid and alkaline hydrolysis ; (2) a similarly stable bonding with the ϵ-amino groups of lysine ; (3) an acid-labile bonding with cysteine with the probable formation of S-sulphonyl cysteine ; (4) a relatively stable bonding with the phenolic group of tyrosine ; and (5) formation of an unstable ring-labelled compound with the imidazole group of histidine. These results suggest that theoretically it should be possible to introduce more fluorescent groups per molecule of protein with DANSYL than with fluorescein *iso*cyanate. The use of other sulphonyl chlorides for fluorescent labelling can be confidently expected and the original Coons' reagent will undoubtedly be reinforced, in the near future, by the use of DANSYL and other sulphonyl chlorides such as the rhodamine derivative whose preparation has recently been described by Chadwick *et al.* (1958).* Their method, with additional notes, is given in the Appendix to this chapter.

A third alternative to fluorescein, also suggested by Clayton and used in the work mentioned above, was described as Nuclear Fast red (benzaldehyde-6-nitro-2-sodium diazotate). As stated, the composition of this compound closely resembles that of Nuclear Fast red salt B or Kernechtrotsalz B (5-nitro-2-amino methoxybenzene diazotate), but not that of Nuclear Fast red (Kernechtrot, Bayer), which is an anthraquinone mordant dye and not a diazonium salt.

* Other fluorescent labels, developed after this chapter was written, are considered in Chapter XXVI.

Neither Kernechtrotsalz or Kernechtrot produce a useful fluorescence when coupled to or adsorbed on proteins, although the second does give a faint yellowish-red fluorescence at times. Although, as Coons (1956) has remarked, two fluorescence colours would be a great advantage in many studies, I can neither understand nor recommend the above method.

Another alternative and contrasting label to fluorescein *iso*cyanate has been prepared and used successfully by Silverstein (1957). This author prepared the nitro derivative of the xanthine dye Rhodamine B by condensing two equivalents of *m*-diethylaminophenol with one equivalent of 4-nitrophthalic anhydride. This process is followed by catalytic reduction to the amine, and this product, on treatment with phosgene, yields the *iso*cyanate. The latter is coupled to protein in the usual way. In his original note on the use of rhodamine *iso*cyanate Silverstein gives no details of the purification of conjugates, and Hiramoto *et al.* (1958), who used this compound for the demonstration of antithyroid antibodies in human sera from cases of chronic thyroiditis, also gave no technical details. It remains to be seen whether the *iso*cyanate or the sulphonyl chloride of rhodamine is better in actual practice.

Alternative Non-fluorescent Labels. Strictly speaking these have no place in a chapter on fluorescent antibody methods, but it is convenient to enumerate them at this point. As suggested earlier in this chapter, the use of *direct colour labelling* has usually proved inadequate for microscopy though it may give satisfactory results on the macroscopic scale. Smetana (1947) and Kruse and McMaster (1949) both used coloured azoproteins as tracer antigens, and the latter showed that diazotized Eeht saure blau and Evans blue could be coupled to proteins in a firm union to give a product visible microscopically when concentrated in such sites as the Kupfer cells.

Of the two alternatives which remain the first, *chromogenic labelling*, was attempted by Mayersbach and Pearse (1956) with little success. A naphthaldehyde conjugated egg white or whole serum was prepared by coupling with 3-hydroxy-2-naphthaldehyde (see Chapter V, p. 88, and Appendix 5, p. 794) at pH 10. Freeze-dried tissues containing the labelled egg white were treated with a diazonium salt at pH 7·4, but the colour developed (a brownish-red) was too weak to demonstrate any but the most concentrated sites (e.g. Kupfer cells). After chromatographic separation of the labelled whole serum, however, the *albumin* fraction gave a strong black colour on treatment with the diazonium salt suggesting that the principle of chromogenic labelling is at least worthy of further trial.

The final alternative is *isotope-labelling* followed by autoradiography. This principle was used by Pressman, Hill and Foote (1949) as part of a long series of studies on ^{131}I-labelled antisera. Clayton and Feldman

(1955) also made use of this method of labelling in their work on the localization of antigenic components of mouse embryos. Many authors have shown that ^{131}I can be attached to proteins without alteration of their immunological specificity (Eisen and Keston, 1949 ; Pressman and Sternberger, 1950 ; Masouredis, Melcher and Koblick, 1951), and that the label remains stable during antigen-antibody reactions (Masouredis, Melcher and Shimkin, 1953 ; Pressman and Keighley, 1948). The distribution of intravenously administered ^{131}I-labelled γ-globulin containing antibodies to thirteen different rat organs was studied by Bale and Spar (1954) and by Spar and Bale (1954). Radio-autographic examination showed the maximum localization of all types of antibody in the renal glomeruli and in the zona reticularis of the adrenal.

Preparation of Antisera. As Coons (1954, 1956) has emphasized, the production of antisera depends on methods which are largely empirical and, for those who are unfamiliar with immunological technique some assistance will be needed. The necessary methods, and the principles behind them, are dealt with by Kabat and Mayer (1948) and by Boyd (1943).

The preparation of the antigen itself may not be entirely straightforward, especially if tissue antigens are being used. Many authors (e.g. Cruickshank and Hill, 1953) have used crude homogenates of single organs or tissue components. These will certainly give rise to multiple antibodies. Vaughan and Kabat (1954), for instance, found an antibody in anti-egg albumin sera which was only revealed by the agar diffusion technique. They showed that the specific antigen was present in a concentration of less than 1 per cent. of the total protein and that it was not detectable by the usual methods of purification, such as electrophoresis. The antibody response to such an antigen may often be disproportionately high.

The use of adjuvants is recommended for increasing the antibody response, especially to non-living antigens. For this purpose the method of Freund (Freund and McDermott, 1942) has most often been used.

Fluorescent Labelling and the Properties of the Conjugates. The process of labelling with fluorescein *iso*cyanate in acetone (or acetone/dioxane 1 : 2) is simple and trouble free. Usually a rough globulin fraction obtained by half saturation with ammonium sulphate is treated with the *iso*cyanate at pH 9 for 18 hours in the cold. The optimum conditions for conjugation were set forth by Coons and Kaplan (1950), who tested the effect of different protein-dye ratios and found that the best results were obtained with about 0·05 mg./mg. protein calculated as fluorescein amine. Similar conditions are used for DANSYL conjugation, except that the necessary concentration of organic solvent can be as low as 10 per cent.

The mechanism of conjugation of proteins and *iso*cyanates was discussed by Hopkins and Wormall (1933), who showed that 80–90 per cent. of the free amino groups of caseinogen disappeared after treatment with phenyl *iso*cyanate at pH 7–8. Chemical and immunological evidence suggested that combination with the ϵ-amino groups of lysine was responsible. If this is true (as seems likely) consideration of the pH range of dissociation of this grouping (Table 6, p. 84) indicates that conjugation should be better at a somewhat higher pH. Coons, in fact, uses pH 9 for the purpose. Failure to form phenylcarbamido compounds with zein (a protein deficient in lysine) was noted by Hopkins and Wormall, but Creech and Jones (1941) were able to obtain slight conjugation with this protein. Some terminal α-amino groups are presumably responsible for this effect.

It has already been noted that the procedures described above result in the attachment of about two molecules of fluorescein per molecule of protein, and attempts to introduce a greater number have not been successful. Schiller, Schayer and Hess (1952–53) labelled bovine serum albumin with increasing amounts of fluorescein *iso*cyanate, but found that at a concentration six times that normally employed the number of groups per molecule only rose from 1·4 to 1·9. These authors made a comprehensive study of the physical and immunological properties of fluorescein carbamido-proteins and concluded that they did not differ in size, shape or homogeneity from the uncoupled proteins.

Purification of Conjugates. Crude fluorescein-labelled globulins produce fluorescence in many structures in normal animal tissues, especially in elastic tissue and endothelium of the blood vessels, in eosinophil leucocyte granules, and in a number of other sites. Non-specific "staining" of this sort is due to at least three factors: (1) fluorescein derivatives bound to globulin and irremovable by dialysis; (2) serum proteins capable of reaction with tissue components; (3) antibodies to such tissue components developed in the course of production of the required antibody.

The first factor is probably responsible for the commonly observed staining of elastic tissue, but Mayersbach and Pearse (1956) have shown that the normal mechanism by which dyes (including fluorescein) stain this tissue is not operating. Previous blocking by means of phenols or naphthols, which prevents the phenolic groups from attachment to dyes by hydrogen bonding, completely failed to prevent non-specific staining by fluorescein-protein conjugates. This first factor, and the second, which are invariably present in the crude conjugates, can be removed by absorption with tissue powder. This empirical stage in the purification of conjugates, which often has to be carried out repeatedly before it is successful, has recently been partly replaced by the substitution of suitable Dowex resins.

Reactions due to the third factor can also be abolished by absorption of the conjugate with a suitably chosen tissue powder, but this process may defeat its object when antigenic components of normal tissue are being studied.

In such a case Coons advises the quantitative absorption of aliquots of the conjugate with assayed fractions containing widely different amounts of the antigen under study. The smallest amount of these fractions necessary just to abolish the observed fluorescence in the tissue section should run parallel with the amount of antigen present in the fractions.

Controls of specificity are absolutely necessary. When a foreign substance is concerned the corresponding normal tissues are a sufficient control for the purpose, but it is wise also to show that the fluorescent reaction can be blocked (reversibly) with the unlabelled specific antibody. As an alternative the conjugate can be treated with the specific antigen until complete absorption of the labelled antibody has occurred.

Conjugates prepared from DANSYL produce much less non-specific staining than do fluorescein conjugates. In many cases it is possible to avoid absorption with powdered tissues or resins entirely. This is a very great advantage from the technical point of view.

Preparation of Tissues. Two types of tissue section can be used for fluorescent antibody studies. These are (1) freeze-dried and (2) fresh frozen sections. The latter are usually produced with a cold microtome or cryostat, but cold knife sections have been used for the purpose. In both cases the necessary prerequisite is satisfied, that the tissue proteins (antigen or antibody) remain substantially unaltered as far as their immunological properties are concerned. Coons and his associates have used the modified Linderstrøm-Lang cryostat for the preparation of sections in their extensive studies, while Marshall (1951, 1954), on the other hand, used freeze-dried sections entirely.

The second method of preparation is undoubtedly the most convenient, since large numbers of sections of many tissues can be prepared in a short time and, provided sections 5–8 μ or thicker are acceptable, it is perfectly satisfactory. Unavoidable artifact is greater than with freeze-dried sections, but quite acceptable from the histological point of view. Although sections thinner than 5 μ can be cut with the cold microtome, nevertheless, if 1–2 μ sections are required so that localization can be made on the intracellular level, freeze-dried materials are better employed. Mayersbach and Pearse (1956) suggested that freeze-dried deparaffined sections were ideal for fluorescence microscopy. Their primary (auto) fluorescence corresponds exactly to that seen in fresh frozen tissues, and there is no evidence to suggest that the short exposure to heat during paraffin embedding has any adverse effect

on the immunological properties of the proteins. There is no question, however, that the physical properties (such as solubility) of many proteins are radically altered by freezing and drying, and Mayersbach (1957b) has found that the preservation of freeze-dried DANSYL-labelled protein in liver sections differs radically from that found in frozen sections and depends on the amount of rehydration which is allowed to occur before the fixative is applied.

The most popular method of fixation, for both freeze-dried and cold microtome or cold knife sections, has been ethanol (85–95 per cent.) or absolute methanol. In the case of viruses whose antigenicity is destroyed by these two fixatives, absolute acetone is used instead. In contrast with the usual practice Marshall (1954) used buffered formaldehyde in dioxan for his work on the localization of enzymes by the fluorescent antibody technique. Some antigens can withstand prolonged formalin fixation and remain antigenic enough to be demonstrated in routine paraffin-embedded sections. These are exceptions, however, and for most work short precipitation of the protein in the sections with ethanol is all that is required.

Fluorescence Microscopy. This differs in no respect from the requirements of ordinary fluorescence microscopy with fluorochromes except that the absolute amount of fluorescent material is small and an intense source of UVL is necessary in order to achieve the maximum degree of excitation. Considerations of the necessary apparatus for microscopy and photomicrography appear in Chapter XXVI, p. 730. Coons (1956) has pointed out that the success of his method, so far as microscopy is concerned, is primarily due to the brilliance with which fluorescein fluoresces. The quantum efficiency of this dye is between 75 per cent. and 85 per cent., and its absorption is from 4,400 Å to 5,200 Å with a maximum at 4,940 Å. The wavelength of the emitted light is from 5,100 Å to 5,900 Å with a peak at 5,180 Å. Since any absorbed light is converted to fluorescence with 75–85 per cent. efficiency it is obvious that the greater the amount of light of wavelength between 4,000 Å and 5,200 Å which is allowed to fall on the stained tissues the greater will be the emission. The quantum efficiency of 1-dimethylaminonaphthalene-5-sulphonic acid has been given by Teale and Weber (1957) as 53 per cent., while for 1-dimethylamino-naphthalene-7-sulphonic acid the figure is 75 per cent.

Applications of the Method

Detection of Foreign Antigens. The greater part of the work done in this category has been carried out on *viruses*. Bacterial infections, presumably because of the adequacy of older techniques in this instance, have been largely ignored. Four papers describe the localization in the tissues of mumps antigens (Coons, Snyder, Cheever and Murray,

1950 ; Chu, Cheever, Coons and Daniels, 1951 ; Watson, 1952a and b). The virus was found by Coons and his collaborators in the cytoplasm of the acinar and duct cells of the parotid gland, and in extracellular sites in the brain and elsewhere. Influenza virus infection was investigated by Watson and Coons (1954), and by Liu (1955a and b) in the chick embryo and in the ferret. In the nasal mucosa of the ferret the antigen was found in the cytoplasm of the ciliated cells, and in both species it was observed in the nuclei (see below, p. 148). Varicella virus was studied in infected cells in tissue culture by Weller and Coons (1954). These authors used a layering technique with human convalescent serum as the intermediate and fluorescent anti-human γ-globulin as the demonstrator. The same technique was used by Liu and Eaton (1955) to demonstrate the virus of atypical pneumonia in the bronchial epithelium of infected chick embryos.

Canine infective hepatitis was studied by Coffin, Coons and Cabasso (1953), and canine distemper by Moulton and Brown (1954) and Moulton (1956). The last of these papers dealt with the process of demyelination occurring in distemper and described the localization of specific fluorescence in the cerebellar white matter, where it was principally confined to the astrocyte nuclei. Not surprisingly, some difficulty was experienced in distinguishing astrocytes from other glial cells. The development of vaccinia virus in human epithelial cells was studied by Noyes and Watson (1955), who showed that the antigenic material, at first finely particulate, was distributed in the cytoplasm in close association with the nuclear membrane. Later, collections of antigen were observed in the nucleus. Noyes (1955) demonstrated the neurotropic Egypt 101 virus (West Nile Fever) in cells cultured from a squamous carcinoma of the larynx, and also in infected mouse brains.

Foreign antigens other than viruses introduced into the organism by injection have been traced by the fluorescent antibody technique in a number of instances. The capsular polysaccharides of the pneumococcus, and of Friedlander's bacillus, were traced in this way by Kaplan, Coons and Deane (1950), and by Hill, Deane and Coons (1950). After intravenous administration in large doses the antigens were found in phagocytic cells throughout the body and also in epithelial cells in many organs. They persisted in the former sites for long periods, but disappeared from most epithelial cells within 3 days. After producing an experimental glomerulonephritis in mice by subcutaneous injection of killed *Proteus mirabilis* Wood and White (1956) found antigenic material from the organism in the affected glomeruli. The polysaccharides of *Cryptococcus neoformans* were found by Eveland *et al.* (1957) in uninfected organs such as kidney and lung. The organisms themselves were easily identified in infected tissues by the fluorescent antibody method.

A number of studies have been devoted to the tracing of foreign protein antigen following their parenteral administration in animals. Egg albumin, bovine serum albumin and human γ-globulin were used by Coons, Leduc and Kaplan (1951) in their work on the fate of intravenously injected foreign protein in the mouse. They found that the initial distribution closely resembled that of injected polysaccharides, except that the proteins were detectable only for a few days at most. Waksman and Boeking (1953) obtained similar results in rabbits following intradermal injection, as did Mayersbach and Pearse (1956) after intraperitoneal injection of egg white in the mouse. More recently Cochrane, Vasquez and Dixon (1957) found the specific antigen (bovine serum albumin) in the lesions of experimental serum sickness, and Wollensak and Seybold (1957) have studied the distribution in rat liver and kidney of injected human γ-globulin and serum albumin.

Nuclear Fluorescence. The finding by Coons *et al.* (1951) of specific fluorescence in the nuclei has produced a great deal of speculation. Coons considered that the transfer of the foreign protein from the cytoplasm to the nuclei during freezing (quenching) or storage was unlikely. During the cutting of fresh frozen sections with the cold microtome dislocation of tissue components is sometimes noted, but nothing is seen approaching the regular displacement which would be necessary to produce nuclear fluorescence as an artifact. Such an artifact, in any case, would not occur with freeze-dried material. Thawing of the frozen sections on the slides or coverslip certainly produces some distortion, but translocation of cell components is not noted. Non-specific reactions (staining of the nuclei) can be completely ruled out. It is therefore necessary to assume that many foreign proteins, depending no doubt on their molecular weight and other characteristics, can be transferred from the cytoplasm to the nucleus, *in vivo*, in a short space of time (Fig. 47, p. 148).

Evidence in favour of nuclear foreign antigen was given by Crampton and Haurowitz (1950), who found ^{131}I-labelled ovalbumin and bovine γ-globulin in the nuclear fractions of rabbit-liver homogenates. Schiller *et al.* (1952–53) found, however, that the fluorescein marker of fluorescent ovalbumin failed to appear in the nuclei. Mayersbach and Pearse (1956) confirmed this finding (Fig. 48, p. 148) and assumed, with the former authors, that differential permeability of the nuclear membrane was the only reasonable hypothesis by which to explain the facts. Later studies by Mayersbach (1957a), however, have shown that fluorescein-labelled egg white can be demonstrated in liver cell nuclei for a short period.

Detection of Native Antigens. The first application of the fluorescent antibody method to the localization of native antigens was that of

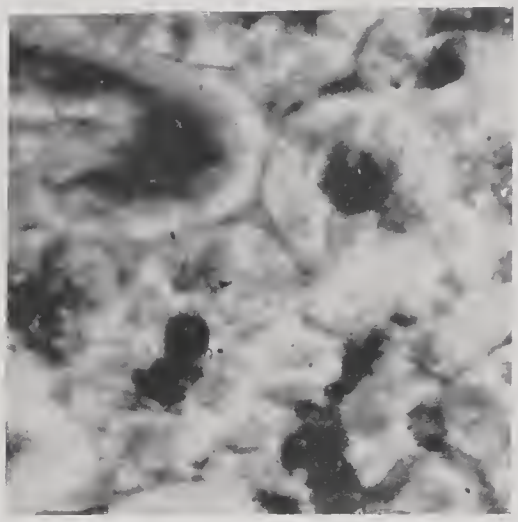


FIG. 47. Mouse kidney. Five μ freeze-dried section. Animal killed 2 hours after egg-white injection. Fluorescent antibody reaction (Fluorescein *iso*cyanate). Nuclei, perinuclear rings and brush borders of the tubules are positively stained. × 800.

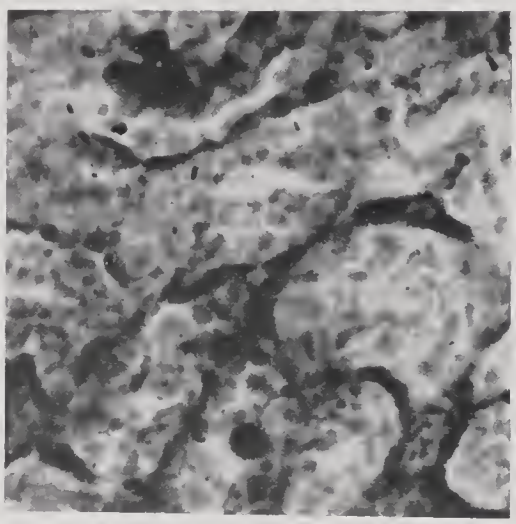


FIG. 48. Mouse kidney. Five μ freeze-dried section. Animal killed 30 minutes after injection of fluorescein-labelled egg white. Strong fluorescence in tubular lumen, brush border, and around the tubular nuclei. × 800.

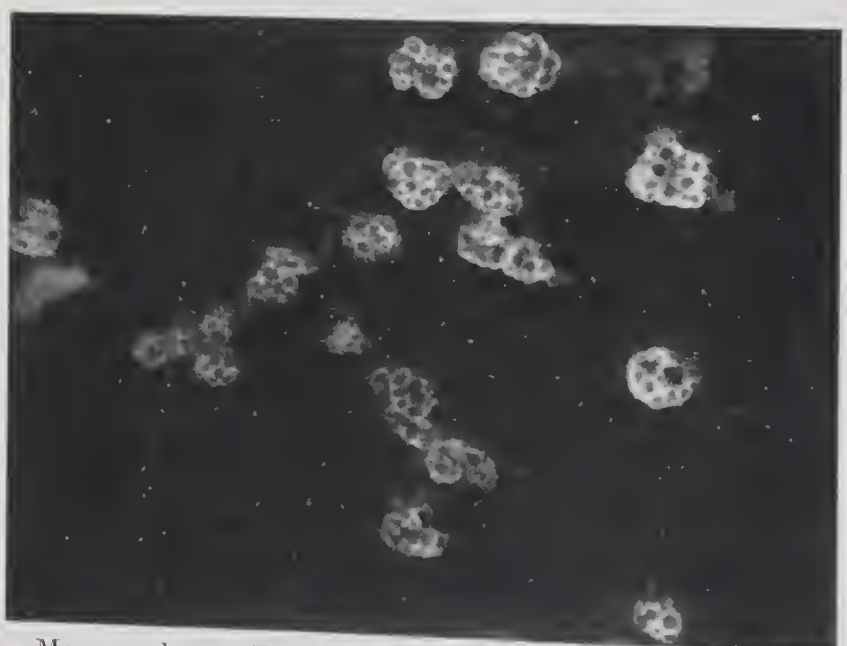


FIG. 49. Mouse spleen. Fresh frozen section from animal immunized with *P. vulgaris* vaccine. Layering technique to show presence of antibody to the organism. (Fluorescein-labelled antibody.) × 600.

[*To face p.* 148.

Marshall (1951), who prepared rabbit anti-pig ACTH and coupled this with fluorescein *iso*cyanate to localize, in the pig pituitary gland, the sites of origin of this hormone. It was found exclusively in cells classed as basophils by other techniques. Marshall controlled his observations by showing that the fluorescent conjugate would precipitate active ACTH from a commercial preparation, and also that it would not stain any cells in the pituitary glands of other species. Later work by this author (Marshall, 1954) has demonstrated the presence of chymotrypsinogen and procarboxypeptidase in the zymogen granules of ox pancreas.

In all work depending on antisera produced in response to crude or even " pure " proteins some means has to be found for dealing with the unwanted antibodies which may be present. Removal of non-specific coupling activities by extraction with liver powder and Dowex resins leaves them substantially unaffected. It is often necessary, therefore, to precipitate a specific component of the antiserum by means of one antigen leaving another in the supernatant. Hill and Cruickshank (1953) described the presence of two antibodies in anti-rat kidney serum, one which would react with the tubular epithelium and another which reacted with glomerular and tubular basement membrane, and with reticulin in blood-vessel walls. Later work (Cruickshank and Hill, 1953) indicated that anti-rat glomerular globulin (containing only the second antibody) and anti-lung globulin stained basement membrane and reticulin in a similar manner. The authors suggested that the antigen in this case was mucopolysaccharide in nature.

In order to establish whether the overlap of antigenic components between glomeruli and other tissues is complete or partial Scott (1957) compared the reactivity of an antiserum against glomeruli with one prepared against synovium. Cross-inhibition experiments showed that reticulin and basement membrane are antigenically distinct ; only reticulin antigen was found in synovium and only basement membrane antigen in the media of arterioles. Preliminary investigations have shown that glomerular basement membrane antigen is present in the choroid plexus, the intrahepatic bile ducts, the thyroid gland and in the media of muscular arteries and arterioles. It is not present in the media of elastic arteries. The reticulin antigen, on the other hand, is found in the same sites as argyrophil reticulin. Another approach to the problem has been that of Mellors and his associates (Mellors, Siegel and Pressman, 1955), who demonstrated the sites at which antibody globulin was found after its intravenous injection. They used fluorescent chicken anti-rabbit globulin to show that the rabbit anti-rat antibody globulin was fixed in the glomerulus of the injected rat. This experiment differs from those of Cruickshank and Hill in revealing only the actual, and not the potential, sites at which fixation of globulin takes place.

As part of a series of studies under the heading "analytical pathology," Mellors and Ortega (1956) have shown that γ-globulin (demonstrated by means of fluorescent rabbit anti-human γ-globulin) is present in the glomeruli in lipid nephroses in childhood and in the same situations in varying types of glomerulonephritis, and in renal amyloidosis. They also found it in periarteritis nodosa. Vasquez and Dixon (1956) have also demonstrated the presence of γ-globulin in amyloid deposits by a similar technique. Further studies by Ortega and Mellors (1957) have revealed that γ-globulin is normally present in human tissues in the cytoplasm of three distinct cell types. These are : (1) immature and mature plasma cells with basophilic cytoplasm ; (2) plasma cells containing Russell bodies ; and (3) certain cells in the germinal centres of the lymph nodes (in the cytoplasmic granules only).

An interesting application of the fluorescent antibody method by Weiler (1956) confirmed an already established antigenic difference between the cytoplasmic particles from normal kidney and those from a stilbœstrol-induced carcinoma. The literature on the investigation of various protein components of the tissues and their distribution in pathological lesions has been added to by the work of Craig and Gitlin (1955), who showed the presence of fibrin in arterial fibrinoid lesions, and of Finck, Holtzer and Marshall (1956), who studied the distribution of myosin in glycerol-extracted muscle fibres.

Detection of Antibodies to Foreign Antigens. Studies in this category are usually carried out by means of layer technique, but, as an alternative, fluorescent specific anti-globulin is used. Although at first sight the use of fluorescent antigen would appear to be ideal, this is not the case in practice. Antigens possess a large number of immunologically active groups by comparison with antibody and the detection of antibody is therefore much less sensitive than the detection of antigen. The problem can be overcome, as described earlier in this chapter, by placing a layer of unlabelled antigen between the unlabelled (tissue) antibody and the fluorescent localizing antibody.

In this way Coons, Leduc and Connolly (1955) demonstrated antibody to ovalbumin or human γ-globulin in plasma cells in the spleen, lymph nodes, ileal submucosa and in the portal spaces of injected rabbits. Lymphocytes never showed any activity. Leduc, Coons and Connolly (1955) investigated the primary and secondary response to diphtheria toxoid and found the former in a few large immature cells in the medulla of lymph nodes draining the injection site. The secondary response was due to the development, supposedly from these immature cells, of colonies of antibody-containing plasma cells. White, Coons and Connolly (1955a) showed that the adjuvant effect of aluminium phosphate was due to delay in absorption of the antigen injected with it from the local site of injection. Prolonged

stimulation of cells in the draining lymph nodes was thus possible. These same authors (1955b) injected guinea pigs with an oil-water emulsion of ovalbumin containing a wax fraction from *Mycobacterium tuberculosis*. The wax stimulated a macrophage response with granuloma formation, but the macrophages contained no antibody. A later plasma cell response produced antibody in large amounts.

A study of the contribution of Russell bodies to antibody formation was made by White (1954). Following the injection of rabbits with *Proteus vulgaris* vaccine large numbers of Russell body-containing plasma cells appeared in the spleen. Using the layer technique White showed that these contained antibody, often in the form ofa ring (Fig. 49, p. 148), but he was unable to show that either Russell bodies or plasma cells contained any antigen.

The relationship between synthesis of anti-ovalbumin and γ-globulin and the distribution of antibody-containing plasma cells was studied by Askonas and White (1956), and Mellors, Arias-Stella, Siegel and Pressman (1955) demonstrated antibodies in the focal lesions of myocarditis and glomerulonephritis in rabbits receiving intravenous foreign proteins. They used fluorescent chicken anti-rabbit globulin for the purpose.

The distribution of γ-globulin in lupus erythematosus was investigated by Mellors, Ortega and Holman (1957). These authors described " staining " of the nuclei of white cells in material from a case of this disorder when fluorescent anti-human γ-globulin was applied. Holborow, Weir and Johnson (1957) used a layer technique in their studies on the LE phenomenon. They treated sections of normal tissues with LE-positive serum and followed this with fluorescent anti-γ-globulin. Once again the specific fluorescence was found in the nuclei of a variety of organs. Control sections untreated with serum, and sections treated with normal serum, failed to show this effect. The production of autoantibodies to normal spinal cord material in rabbits was demonstrated by Beutner *et al.* (1958) who found that the main antibody combining site was in the myelin sheaths.

Conclusion. The very great increase in the number of studies made in the past six years by means of the fluorescent antibody technique indicates that most of the technical difficulties have been overcome. Applications to pathological problems which have so far been recorded represent a beginning from which great advances may be expected to stem.

Since fluorescein *iso*cyanate is unstable, and since difficulty in obtaining it represents one of the bigger stumbling blocks to wider application of the method, the possibility of producing stable dry fluorescent antibody protein by freeze-drying should be investigated. If purified dried conjugates became available (fluorescein *iso*cyanate,

rhodamine *iso*cyanate, rhodamine sulphonyl chloride, or DANSYL) a considerable widening of the field of application of the Coons' method might be expected, especially in human pathology.

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CHAPTER VII

THE HISTOCHEMISTRY OF SOME IMPORTANT SIMPLE PROTEINS

The purpose of this chapter is to describe the application of modern histochemical techniques to the differentiation of various simple protein structures. In some cases this has been of academic, but in others of strictly practical use. The methods set forth in Table 10, p. 162, were chosen simply to answer the various physical and chemical questions as they arose. They are by no means a comprehensive list of all the available methods but may serve to indicate the manner in which problems concerning these tissue materials may be solved. Although microstructure in the molecular range can only be resolved by the physical techniques of X-ray diffraction and electron microscopy, it is well to remember that both these techniques have their limitations when applied to histological material in its complete state. Between the molecular level at which these physical methods operate and the microscopical level of ordinary morphological histology there lies a realm of submicroscopical structure which can be reached by histochemistry.

Collagen and Reticulin

Histologically collagen and reticulin are two easily distinguishable substances, collagen being taken to mean coarse non-branched fibres which are birefringent, red with van Gieson's stain, faintly pink with PAS, blue with sulphation-metachromasia, and yellow or brown with silver impregnation techniques. Reticulin, on the other hand, is taken to mean fine branching fibres which are usually isotropic in paraffin sections, unstained or faintly pink with van Gieson, magenta with PAS, red with sulphation-metachromasia, and black by silver impregnation. On the important question of the relation between these two substances, three views have been expressed by various authors : (1) Collagen and reticulin are one and the same. (2) They are the same chemically but differ physically. (3) They differ both physically and chemically.

As observed by Lillie (1945, 1952a and b), a wide variety of acid dyes, used at moderately acid pH levels, show essentially the same specificity for the two substances, though van Gieson's method provides an exception. If the concentration of acid fuchsin is raised, however, reticulin fibres can be stained red without difficulty. Methods based on the use of acid dyes therefore indicate a substantial similarity

between the two. Mallory and Parker (1927) observed that when certain connective tissue fibres occurred singly they gave the staining reactions of reticulin but that on joining a bundle of fibres they stained as collagen. For this reason these authors considered collagen and reticulin to be identical. Foot (1925, 1927), however, described reticulin as the precursor of collagen and the latter as a chemical substance in the tissue juices which impregnated reticulin fibres. He discarded the unitarian view mainly on evidence derived from his silver staining method, and was unable to demonstrate reticulin fibres arising directly from cell bodies as others claimed to have done. Heringa and Weidinger (1942) and Dublin (1946) also believed that reticulin was "precollagen," in the sense of forming a scaffold, and that collagen was a chemical substance which impregnated reticulin fibres. Lillie (1947), using his periodate-nitric acid-Schiff (periodic acid-Schiff) method, succeeded in obtaining a deep staining of the reticulin network of a variety of organs and the basement membranes in some of those studied. Collagen was only faintly pink. This new evidence was taken to indicate that collagen and reticulin are not chemically identical. Lhotka and Davenport (1950) compared Foot's silver method with the periodic acid-Schiff (PAS) method and found identical histological pictures in a number of tissues. They suggested that the histochemical bonding which occurs in the two procedures is conditioned by the occurrence of similar free radicals in the loci that are stained, and furthermore that these radicals might be aldehydes.

A similar view was expressed by Glegg *et al.* (1953), who showed chromatographically that reticulin contains galactose, glucose, mannose, fucose and ribose in large quantities while collagen contains very small amounts indeed. Bangle and Alford (1954), by means of the anthrone reaction, found 0·55 per cent. of carbohydrate in collagen (commercial hide powder), whereas Windrum *et al.* (1955), with the same reaction, found an average of 4·2 per cent. non-hexosamine carbohydrate in their samples of reticulin. In view of the fact that hide powder contains reticulin and ground substance as well as collagen it is probable that the carbohydrate value for the latter is much lower than 0·55 per cent. In a combined histochemical and chromatographic study Glegg *et al.* (1954) showed that connective tissues in general possessed two carbohydrate fractions, the first rich in glucosaminic acids and the second in aldoses. Most tissues contained more of fraction II, but cartilage contained 14·8 per cent. of its dry weight as fraction I, and 3·5 per cent. as fraction II. The sulphation-metachromasia method of Kramer and Windrum (1953, 1954) (see Chapter IX, p. 254) reflects the high aldose content of reticulin. This component stains red by the method, while collagen remains blue. The absence of galactosamine from reticulin excludes the possibility that it is a complex of collagen-type fibrils with

chondroitin, and the absence of uronic acid rules out a similar complex with hyaluronic acid.

The *in vitro* formation of fibrils by the addition of various acid mucopolysaccharides to solutions of collagen (Gross *et al.*, 1952 ; Morrione, 1952 ; Zawisch, 1957) may reflect the necessary *in vivo* conditions for the formation of this component. It has little bearing on the formation of reticulin.

The question of a cementing substance not only in basement membranes, but holding together the individual fibres of connective tissue, has been considered since its original description by Rollett (1871) and by Flemming (1876), who both stated that such a cementing substance occurred and also that it was of a mucinous nature. In more recent years the view arose that the mucinous ground substance of the tissues was the acid mucopolysaccharide hyaluronic acid, present in loose combination with protein substances. The specific enzyme, hyaluronidase, releases this substance from combination in the tissues. McClean (1931, 1936) observed that hyaluronidase had no direct action on collagen fibres, although swelling and splitting of the fibres was noted. This suggested some action on an interfibrillary cement substance. Bensley (1934) reported the observation of a layer of mucoprotein surrounding collagen fibres in tendon, and Robb-Smith (1945), using streptococcal and testis hyaluronidase, produced a separation of the reticulin membrane of muscle from the underlying fibre, which he attributed to removal of a hyaluronic acid-containing cement. He found no action on collagen and reticulin with either of the two hyaluronidases mentioned, or with *Clostridium welchii* filtrates. Day (1947) confirmed the existence of a cementing substance holding together the individual fibrils of loose connective tissue, but did not consider it to be mucinous. He treated unfixed connective tissue with both testicular and streptococcal hyaluronidase without causing any alteration in its properties. A solution of trypsin, however, acting at 20° for 24 hours, caused loss of coherence and elasticity and Day considered this to be due to removal of a proteinous cement-substance. Aikat and Dible (1956), using *Cl. welchii* filtrates as their source of hyaluronidase, have shown that collagen bands, staining brown with ammoniacal silver techniques, can be split into individual black-staining reticulin fibrils by its action. This last evidence supports the conception of a mucopolysaccharide-containing cement-substance, depolymerized by hyaluronidase.

Physical Chemistry of Collagen

Collagen was regarded by Schmitt, Hall and Jakus (1942) as a scleroprotein which when in sol form exists with its individual molecules widely separated. When reticulin fibres appear in this sol they do so

by arrangement of the molecules into micellar form, that is to say, into aggregations of molecules orientated in a particular manner. Collagen in gel form was regarded as a further condensation of the scleroprotein molecules. According to Astbury (1940) X-ray diffraction studies indicated that collagen fibres are composed of fully extended chains of C and N atoms held together by lateral bonds of an electrovalent nature. These linkages are comparatively strong but easily penetrated by ions. Later studies of the high-angle X-ray diffraction pattern of collagen fibres, and the interpretation of their results, are given by Cowan *et al.* (1953, 1955) in the proceedings of two valuable symposia, to which the reader is referred. Differences in the pattern given by collagen and reticulin were ascribed by Little and Windrum (1954) to the presence of amorphous material in the latter.

Studies with the electron microscope by Kramer and Little (1953) and by Irving and Tomlin (1954) have shown conclusively that the characteristic fibril of collagen, with its 650 Å cross-striation, is present also in reticulin. Kramer and Little, however, found that whereas collagen is composed of bundles of regularly arranged fibrils, reticulin has a membranous structure in which the fibrils occur in a disorderly manner. They concluded that reticulin differed from collagen in four respects : (1) in having an abundant carbohydrate-rich, amorphous matrix ; (2) in having randomly arranged fibrils ; (3) in its membranous structure ; and (4) in failing to yield gelatin on boiling. They compared collagen and reticulin to rope and linoleum, and this seems to be an excellent analogy, as far as the purely static picture is concerned.

A number of authors, myself included, have stated erroneously that reticulin is not birefringent. Brewer (1957), confirming the earlier observation of von Mollendorf (1932), finds that while collagen shows positive form and positive intrinsic birefringence, reticulin possesses positive form and negative intrinsic birefringence. When stained with certain dyes collagen shows dichroism and anomalous colours (see Chapter X, p. 284), whereas reticulin does not. Brewer suggests that this is because its side chains are not free to combine with dye molecules, and, in confirmation of this thesis, he finds that blocking the side chains of collagen, by tanning, prevents the development of dichroism and anomalous colours. Missmahl (1957) has also reported some studies on the birefringence of reticulin.

Biochemistry of Collagen

Accurate figures for the amino-acid composition of native collagen and of the product of its partial hydrolysis (gelatin), are available and these show a high content, particularly, of glycine, proline and hydroxyproline. Bowes and Kenten (1949) estimated the relative amino-acid

contents of collagen and reticulin by judging the intensity of the spots in paper chromatograms of hydrolysates of the two substances. They found the amino-acid distribution to be similar in both except for proline and hydroxyproline in which reticulin was somewhat deficient. Windrum *et al.* (1955), however, made a complete amino-acid analysis of reticulin and compared this with the figures of Bowes *et al.* (1953) for collagen. From a comparison such as this, between human reticulin and bovine collagen, conclusions must be made with circumspection. It seems that the differences between the two, even in respect of proline and hydroxyproline, are scarcely significant.

Jackson (1957) made a biochemical study of the formation and removal of collagen following injection of carrageenin. This is a sulphated polygalactose extracted from the seaweed Irish moss (*Chondrus crispus*), which was shown by Robertson and Schwarz (1953) to produce granulomas rich in collagen when injected subcutaneously into guinea-pigs. Three forms of collagen have been described. One form, which is referred to as acid-soluble collagen, is extractable with dilute acetic acid or with acid buffers. The second form can be extracted from skin with a weakly alkaline phosphate buffer or by means of neutral salts. It is therefore called either alkali-soluble or neutral salt-soluble collagen. These terms are interchangeable (Gross, Highberger and Schmitt, 1955 ; Jackson and Fessler, 1955). Insoluble collagen, the third form, constitutes the bulk of the collagen of the tissues. Between the first and third forms there is no sharp dividing line. It has been shown, for instance, that treatment with hyaluronidase (Chapter XXII, p. 609) increases the acid-soluble form at the expense of the insoluble form.

In the carrageenin granuloma collagen formation reaches its zenith in 7–9 days and resorption apparently commences as soon as this point is reached. On the third day Jackson found 40 per cent. of the neutral salt-soluble form, the remainder being insoluble. After the third day the former fell until the fourteenth day, when it began to rise again. The insoluble form, on the other hand, rose to over 80 per cent. on the ninth day and thereafter fell. Comparison with parallel histological studies carried out by Williams (1957) suggests that carrageenin first stimulated the formation of neutral salt-soluble collagen and then, after the fourteenth day, activated a mechanism leading to the removal of collagen.

Jackson showed that (α-^{14}C) glycine was incorporated into the neutral salt-soluble form of collagen with great rapidity, and into acid-soluble and insoluble collagen much more slowly. His view, in opposition to that of Orekhovitch (1955) is that neutral salt-soluble collagen is the precursor of the insoluble form, and that the latter and acid-soluble collagen are very similar. Harkness *et al.* (1954) suggested that

acid-soluble collagen was derived from recently laid-down collagen and from the outer layers of larger fibres and reticulin fibres. According to Jackson extraction with 0·2 M-NaCl renders reticulin fibres non-argyrophilic and red with acid fuchsin. After citrate extraction argyrophilic fibres are no longer demonstrable. This suggests that reticulin fibres consist of acid-soluble collagen with a coating of neutral salt-soluble collagen not yet incorporated into the fibre structure.

A tentative scheme for the formation of collagen in developing connective tissue was put forward by Jackson (*loc. cit.*). He considers that neutral salt-soluble collagen is secreted by fibroblasts and transformed into collagen in one of three ways : (1) by formation of new fibrils which are argyrophilic and behave as acid-soluble collagen ; (2) by accumulation on fibrils formed as above ; and (3) by direct incorporation into pre-existing insoluble collagen fibres. These views are very interesting and, if they can be integrated with the data derived from other disciplines, a great advance in our understanding of the collagen-reticulin enigma will have been made.

Thermal Shortening of Collagen and Reticulin. The shrinkage of collagen fibres on heating in the presence of water is a well-known characteristic and the process has been studied by most of the physico-chemical techniques mentioned above. The most prominent changes recorded are (1) loss of resistance to trypsin and (2) loss of the optical properties of the fibre. Gustavson (1956) in his book on the " Chemistry and Reactivity of Collagen " deals extensively with the whole theme. If tissues fixed in a variety of aqueous or alcoholic fixatives are subjected to mild heating, subsequent benzoylation no longer prevents the reaction of collagen and reticulin with the coupled tetrazonium reaction (Pearse, 1951). Often the degree of heating necessary to dry the section after mounting on the slide is sufficient to produce this resistance to benzoylation. Exposure to 70°–90° of dry heat, for 5–10 minutes, invariably does so. French and Edsall (1945) stated that formalin fixation increases the temperature at which collagen undergoes thermal shortening from 65°–70° to about 90°. Moreover, the thermal shortening of formalin treated fibres, unlike that of normal fibres, is reversible. The birefringence of collagen in histological sections disappears if the slide is subjected to moderate dry heat (70°–90°) for 30 minutes or more and the colour developed in the tetrazonium reaction after benzoylation becomes deep brownish red instead of purple. These two observations suggest that formalin-fixed collagen can still undergo the same thermal change even when actual visible shortening is prevented by their being fixed on a slide. The process is probably better regarded as a kind of condensation. No difference can be detected in the behaviour of collagen and reticulin, the coarsest and finest fibres staining identically, though the former

are deeper coloured in the aggregate. This evidence suggests physical identity, the application of heat being presumed to cause a structural alteration by which the reactive groups of tyrosine, tryptophan or histidine are prevented from combining with benzoyl chloride, but remain reactive to diazonium salts. As described in Chapter V, many other protein substances, subjected to mild heat, behave in a similar manner so that resistance to benzoylation after heating probably has a physical significance only. Theoretically, that is to say, if collagen and reticulin were entirely different proteins from the amino-acid point of view they might still react in the same way. Further evidence is available, however, which suggests a greater degree of structural identity between collagen and reticulin. This is to be found in studying the effects of mild heat in preventing acetylation of the reactive groups of tyrosine, tryptophan or histidine by acetic anhydride in dry pyridine. Collagen, and to a lesser extent reticulin, remain resistant to acetylation, whereas in many other proteins the specific groups, though resistant to benzoylation, can be successfully acetylated and prevented from giving a positive tetrazonium reaction. Gustavson (1956), using fixation by chromium compounds and condensed naphthalene sulphonic acid as indicators, has shown that thermal shrinkage of collagen does not alter the reactivity of its ionic groups (mainly COOH and NH_2). On the other hand, the denatured product combines far more readily with aggregated chromium compounds which react by coordinate valency forces with the OH groups of the collagen. Gustavson suggests, therefore, that the main change in thermal shrinkage is the rupture of hydrogen bond cross-links. How this could affect the resistance of heated collagen to benzoylation and acetylation is not understood.

The behaviour of tissue proteins in the coupled tetrazonium reaction (Appendix 5), with and without benzoylation and with and without mild heating, is shown in Figs. 50, 51 and 52 and also in Figs. 53 and 54. The first three figures illustrate the staining of human renal tubules containing hyaline droplets : (1) by the tetrazonium reaction alone ; (2) by the tetrazonium reaction after 16 hours benzoylation ; and (3) by the tetrazonium reaction after heating for 10 minutes at 80° (dry) and 16 hours benzoylation. In the first illustration (Fig. 50) all protein-containing structures, including a cast in the lumen of a tubule, are stained with varying degrees of intensity. In the second (Fig. 51), only the nuclei and a few of the hyaline droplets are stained, while a protein cast in the tubule at lower left is colourless. In the third (Fig. 52), all the hyaline droplets are stained and also the protein cast. Figs. 53 and 54 indicate the behaviour of fibrin deposits in the glomerular tuft in a case of cortical necrosis. The first of these two illustrations shows the effect of the tetrazonium reaction

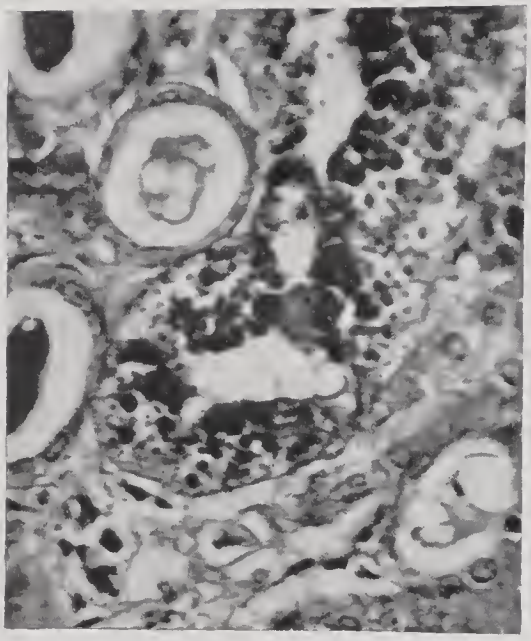


FIG. 50. Formalin-fixed human kidney (5·5 μ paraffin section). All protein structures, including nuclei, cytoplasm, hyaline droplets and tubular casts, are strongly stained. Coupled tetrazonium reaction. × 335.

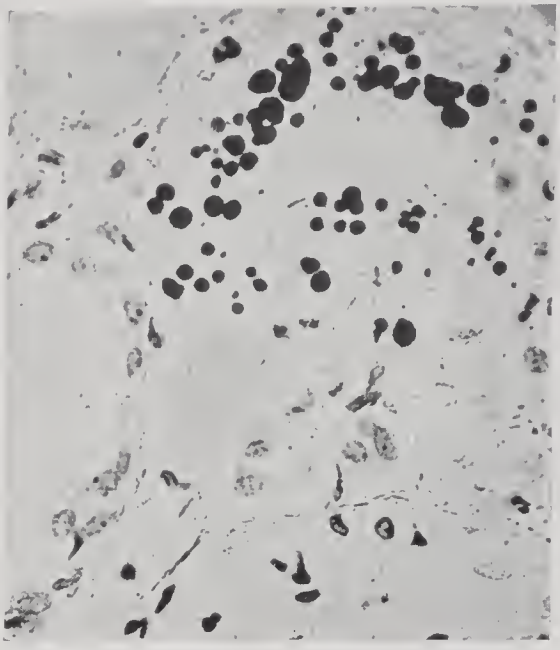


FIG. 51. As Fig. 50, but subjected to 16 hours' benzoylation at 22°. Only the nuclei and some hyaline droplets are stained. The tubule at lower left contains a protein cast. Coupled tetrazonium reaction. × 335.

FIG. 52. As Fig. 51, but subjected to 10 minutes' dry heat at 80° before benzoylation. All the hyaline droplets and the protein casts are now stained. Coupled tetrazonium reaction. × 335.

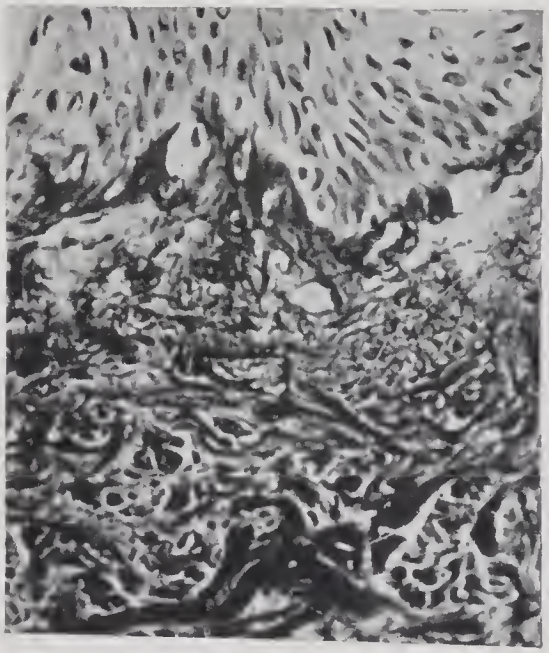


FIG. 59. Human skin (5·5 μ paraffin section). Showing resistance of collagen and reticulin, but not elastic tissue, to the process of benzoylation. Coupled tetrazonium reaction. × 235.

[*To face p.* 160.

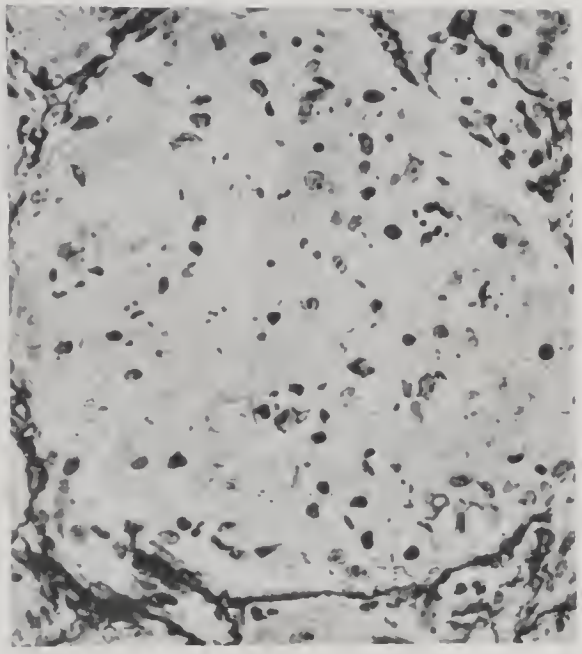


FIG. 53. Human kidney biopsy (cortical necrosis) (5·5 μ formalin-fixed section), showing a single glomerulus. Sixteen hours' benzoylation. Coupled tetrazonium reaction. × 220.

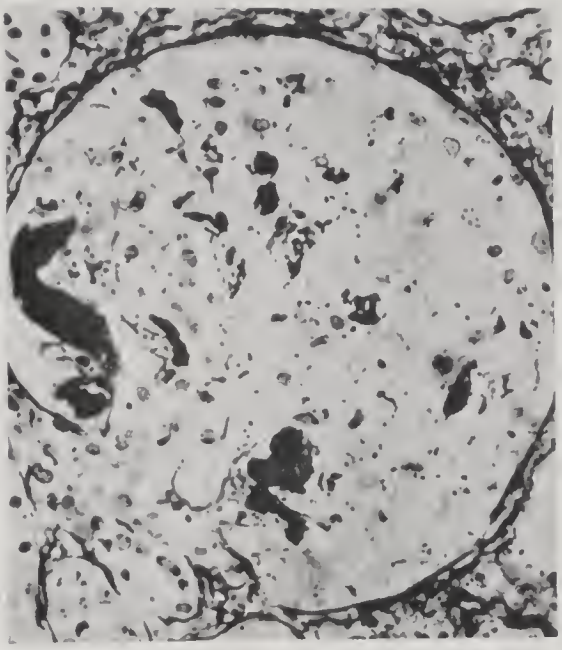


FIG. 54. Serial section to Fig. 53. Subjected to 10 minutes' dry heat at 80 before benzoylation. Fibrin masses in the glomerular tuft are now visible. Coupled tetrazonium reaction. × 220.

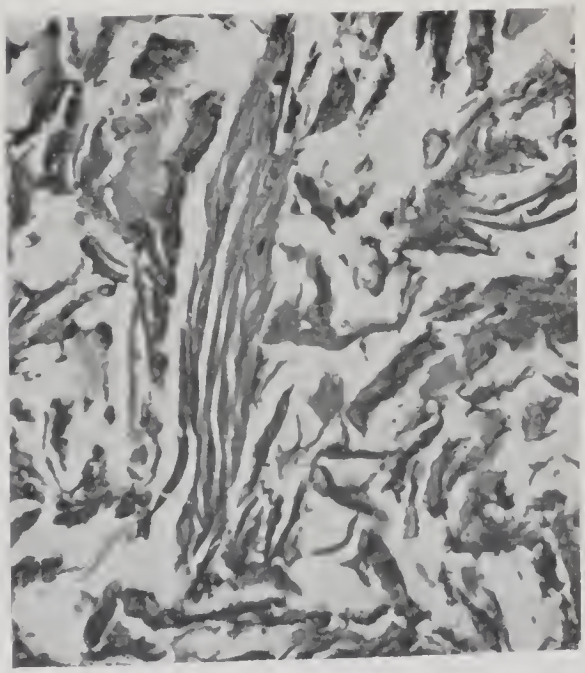


FIG. 57. Keratin flakes in an epidermoid cyst. For comparison with Fig. 58. Hæmalum and eosin. × 350.

FIG. 58. Serial section to Fig. 57. Photographed by polarized light to show plain birefringence. × 350.

after 14 hours benzoylation ; only nuclei and the coarser connective tissue fibres are stained. In the second glomerulus, which was treated in parallel with the first after short heating on the warm stage, fibrin deposits are strongly stained.

Lipids of Collagen and Reticulin. A lipid component of human renal reticulin has recently been described by Windrum *et al.* (1955). This is the saturated fatty acid myristic acid, which is apparently closely bound to protein since it resists extraction with boiling $CHCl_3/CH_3OH$. Solvent extracted reticulin, treated in this way, contains about 10 per cent. of myristic acid, calculated on a dry weight basis. The plasmal reaction (Chapter XII, p. 347) indicates that collagen contains a minimal amount of acetal phospholipids, the substances responsible for the positive reaction with Fuchsin-sulphurous acid in untreated control sections for the Feulgen and PAS techniques. Although chemical assays reveal a small amount of lipid in white fibrous tissue, which presumably includes any plasmalogens if present, this is not revealed by histochemical procedures. The PAS reaction in the case of collagen, since it is resistant to the action of diastase, can therefore be assumed to indicate protein-bound carbohydrate only. In practice, the amount of colour developed in collagen by this reaction varies from pale to deep pink, even when the times of the various stages are strictly controlled, as they must be in comparative work. One assumes from this that the amount of bound carbohydrate in collagen is variable also. The degree of reticulin staining with the PAS reaction, and probably therefore its carbohydrate content, vary less than they do in the case of collagen. Windrum and his colleagues showed that their lipid component was not responsible for the PAS reaction of reticulin, and they made several interesting suggestions concerning the role of myristic acid in pathological processes affecting connective tissues.

Immunological Properties of Collagen and Reticulin. Cruickshank and Hill (1953) showed by means of the fluorescent antibody reaction that rabbit anti-rat glomerular serum contained an antibody which would react specifically with reticulin, basement membrane, sarcolemma and neurilemma, throughout the body. There was no reaction with collagen fibres, elastic tissue or cartilage. The suggestion was made, therefore, that the antigenic properties of reticulin might depend on its polysaccharides. This aspect of the problem has been investigated by Milazzo (1957), who endeavoured unsuccessfully to extract a specific haptenic or antigenic fraction from trypsinized reticulin samples. He concluded that the " antigenic component of reticulin is probably integral with its stable glyco-lipo-protein non-collagenous moiety." It could not be decided, from the work of Cruickshank and Hill, whether reticulin and basement membrane were antigenically identical. Scott (1957), however, has shown that

they are not. He found reticulin antigen in synovia and basement membrane antigen in the arterial media. Glomeruli contain both antigens. Connective tissues must be regarded as antigenically heterogeneous.

TABLE 10

Properties of Various Scleroproteins and Fibrin

Property or test	Frozen or paraffin sections	Collagen	Reticulin	Fibrin	Fibrinoid	Elastic	Keratin
Isoelectric point	...	4·85	...	5·5	...	6·0	...
Mallory PTAH	P	Red-brown	Red, occ. (blue-lined)	Blue	Red-brown to blue	(Coarse) blue (fine) red	Blue
Toluidine blue	F	Variable meta-chromasia	...	Ortho-chromatic	Variable meta-chromasia	Ortho-chromatic	Ortho-chromatic
Sulphation Metachromasia	P	−	+	−	+	−	−
Pseudoplasmal reaction	F	−	−	−	−	+**	−
Lipid (Sudan black)	F	−	−	Variably +	Variably +	−	Outer corneum +
Lipid aldehyde (naphthole acid)	F	−	−	−	−	+**	−
Birefringenec	F	+	+	+ or −*	−	−	+
Autofluorescence	F	−	−	−	−	+†	−
Gram	P	+	−	+	occ. partly +	−	−
Aldehyde-fuchsin	PF	−	−	−	−	+	−
Tetrazonium	P	Red-purple	Red-purple	Deep red	Deep red	Brownish-gold	Brown
Tetrazonium (benzoylated)	P	Red-purple	Red-purple	Deep red	Deep red	−	−
PAS after diastase	P	Variably + (pink)	Variably +	Occ. + (pink)	+ +	+ or −‡	−
Crystalline pepsin	Unfixed	Rapid	Rapid	Rapid	Less rapid	Nil	Nil
Crystalline trypsin	Unfixed	Slow	Very slow	Rapid	Less rapid	Nil	Nil
Silver methods	P	Brown to yellow	Black	−	Yellow to brown	−	−
Acid haematein (Baker)	P	−	−	Pale blue	−	−	−
Sakaguchi reaction	P	Moderate	Moderate	Strong	Strong	Weak	Strong

* Fibrin film is birefringent. Fibrin in the tissues variably so.
** Particularly in rodents.
† Arterial and pulmonary elastic tissue especially.
‡ Positive in human dermis ; weak or negative in most other sites.

At this point is inserted Table 10, which sets forth the various properties both of collagen and reticulin, and of the other scleroproteins which remain to be discussed. It emphasizes the difference between these various scleroproteins and may act as a basis for the correlation of their staining properties with physical and chemical structure.

Conclusions. Very considerable advances have been made in the past five years in our knowledge of the structure of both collagen and reticulin, and the observed differences in staining are reasonably explained by differences in the arrangement of the characteristic

component fibrils and of their matrix or cement. Fig. 55 illustrates these points in diagrammatic form.

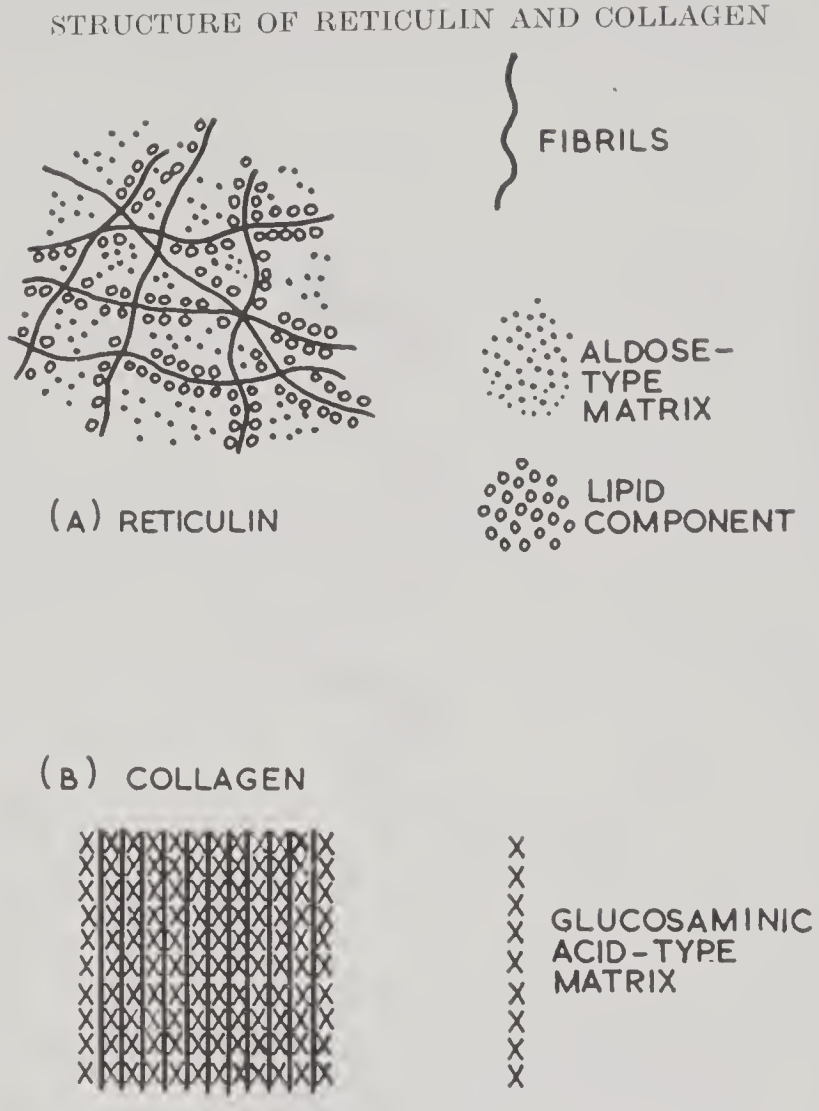
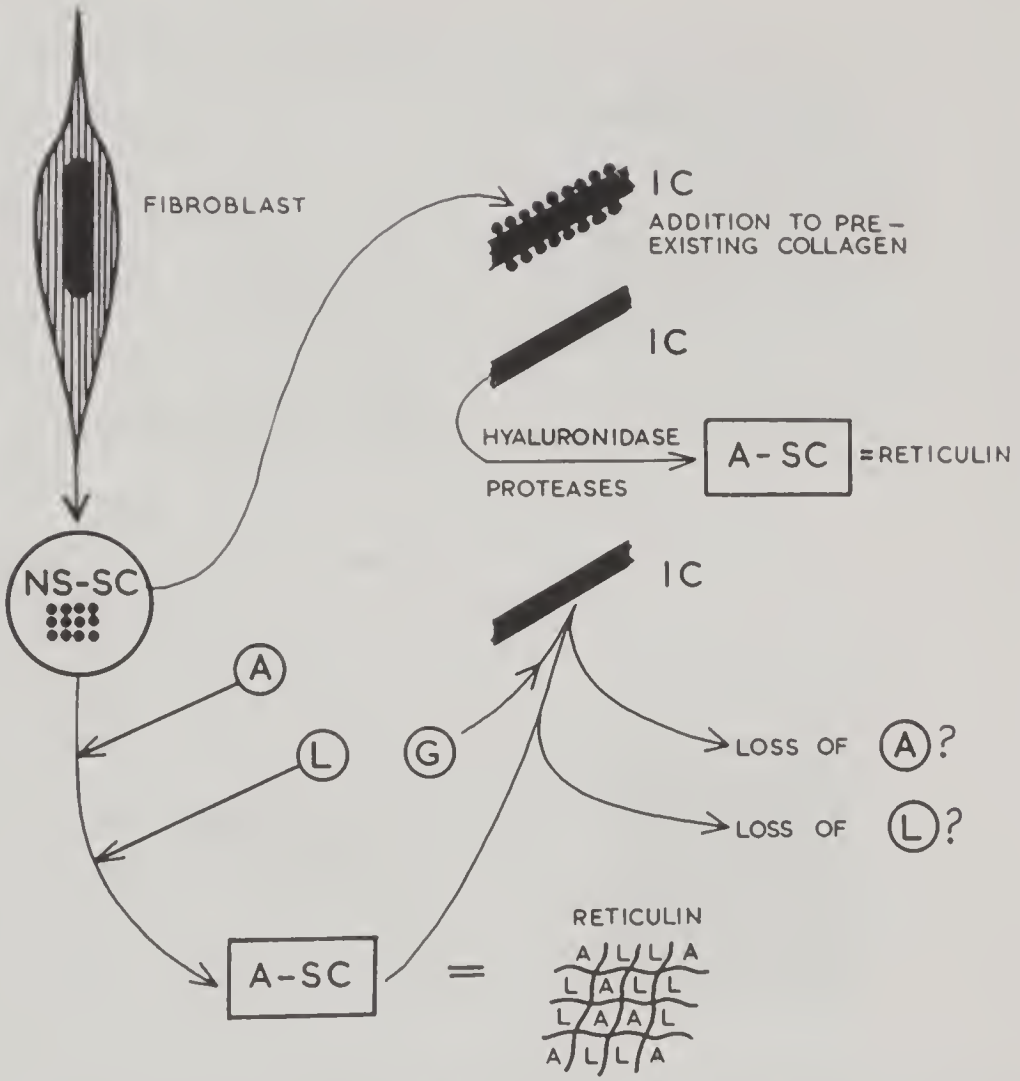


Fig. 55.

If Brewer's (1957) evidence is accepted, the positive form birefringence shown by reticulin indicates that it is made up of longitudinally arranged particles. Since the electron microscope studies of Kramer and Little suggest a random arrangement of fibrils it follows that in most samples of reticulin there is sufficient longitudinal alignment to give the observed birefringence. The negative intrinsic birefringence of reticulin indicates the presence of a component at right angles to the fibre axis, and this is possibly Windrum *et al.'s* myristic acid.

A tentative scheme for the formation of collagen can be drawn up which takes into account most of the biochemical factors discussed above. It serves to emphasize the points which still require clarification. The initial stage of collagen formation involves the production of NS-SC by the fibroblast and its transformation into A-SC or reticulin. We do not know whether myristic acid is present in the fibroblast and

we are fairly certain that the quantity of aldose sugars is small. The addition of these substances to NS-SC, under whatever auspices it takes place, is obviously of vital importance in collagen formation. Although both substances are tightly bound to the protein of reticulin



NS–SC = Neutral self-soluble collagen
A–SC = Acid-soluble collagen
(A) = Aldose
(L) = Lipid
(G) = Glucosaminic acid
I C = Insoluble collagen

Fig. 56.

the process appears to be readily reversible and conversion of A-SC to IC must take place by loss of the aldose and lipid fractions of reticulin. It is possible that these are not so prominent a feature of dynamic reticulin, reticulin that is in the process of conversion. The change-over from one type of matrix to another in the conversion of reticulin to collagen cannot at present be explained.

Elastic Tissue

I have here purposely used the term elastic tissue rather than elastin because it is the structure, rather than the protein material isolated from it by the chemist, in which we are interested. As with collagen and reticulin, from the histological point of view, elastic tissue is easily distinguishable by means of a number of specific stains, some of which are of considerable antiquity. The most important of these stains are the various resorcin-fuchsin methods associated with the name of Weigert, the orcein method of Tänzer (1891), hæmatoxylin methods such as those of Harris (1902) and Verhoeff (1908), and the latest aldehyde fuchsin method of Gomori (1950). Michaelis (1901) showed that a number of basic dyes could be used in place of fuchsin in the Weigert method and that other phenols might be used instead of resorcin. Michaelis also showed that oxidizing agents such as ammonium persulphate might replace the ferric chloride ; he was unable, however, to throw any light on the mechanism of the Weigert stain. Table 11, largely derived from Harms' (1957) review, presents the whole spectrum of elastic tissue staining, historical and present.

TABLE 11

Staining of Elastic Tissues

	Acid Staining
Unna, 1886	0·5 per cent. dahlia in alcoholic HNO_3
Koppen, 1889	5 per cent. crystal violet in 5 per cent. phenol
Tänzer, 1891	1 per cent. orcein in acid alcohol (HCl) pH 1·3
Pranter, 1902	1 per cent. orcein in acid alcohol (HNO_3) pH 0·9
Weigert, 1898	0·25 per cent. resorcin-fuchsin in acid alcohol
Harris, 1901	Mucicarmine with acid alcohol differentiation
Michaelis, 1901	Resorcin and other phenols with fuchsin, safranin, methyl violet, etc.
Verhoeff, 1908	Iron-hæmatoxylin
Matsuura, 1925	Phosphotungstic acid-Congo red
Petry, 1952–53	Phosphomolybdic acid-hæmatoxylin
Gomori, 1950	Aldehyde-fuchsin
	Neutral Staining
Klüver and Barrera, 1953	Alcoholic Luxol fast blue (method only, not application)
	Alkaline Staining
Herxheimer, 1886	Lithium carbonate-hæmatoxylin
Salthouse, 1944	Ammoniacal chlorazol black
Witke, 1951	Alkaline primulin (fluorescence)
Lautsch *et al.*, 1953	Alcoholic chlorazol black-benzazurin

The mechanism by which most of these stains work remains an enigma, but some attempt has been made by Dempsey *et al.* (1952), Weiss (1954) and by Fullmer and Lillie (1956) to explain the nature of orcein staining of elastic tissue. Engle and Dempsey (1954) found that orcein was a mixture of closely related amphoteric dyes which could be separated into four principal coloured fractions. Fractions I and II were cationic, Fraction III anionic, and Fraction IV partly anionic and partly electro-neutral. When these were used to stain formalin-fixed horse ligamentum nuchæ Fractions I, II and III stained certain fibres throughout the pH range up to 9·5, with maximum intensity at pH 3–8·5 for Fraction I, pH 5–7 for Fraction II and pH 2·9 for Fraction III. The authors concluded that orcein staining was relatively insensitive to pH and, therefore, probably due to reaction through non-ionic bonds. Their Fraction II could be used as an acetic acid orcein to stain nuclei blue. Musso (1956), using the technique of distribution chromatography with cellulose or silica gel at pH 11–12, obtained twelve brightly coloured components from samples of orcein. Five of these he was able to crystallize but, because of the small amounts available, none were applied to the staining of elastic tissues. Fullmer and Lillie (1956) were unable to prevent orcein staining of elastic tissue after blocking its carbonyl, hydroxyl, amine and aldehyde groups by a variety of procedures. After such procedures, however, they found that normal collagen would stain with orcein. It was evident, from this work, that polar groups must interfere in some way with orcein staining. Further experiments showed that acetylated or nitrosated (deaminated) orcein could not function as a stain, indicating that the aryl hydroxyl or amine groups of the dye molecule are required for staining. These views are essentially the same as those of Weiss (1954). This author estimated the heat of reaction between elastic tissue and orcein as between — 6,000 and — 6,500 calories per mole. These figures are consistent with a reaction based on the formation of hydrogen bonds. The question as to which group in elastic tissue takes part in hydrogen bonding with the phenolic groups of the dye is less easily answered. Fractions I and II of Engle and Dempsey, which are responsible for "orcein" staining, carry a positive charge in acid solution. They are therefore repelled by the positively charged side chains of most proteins at such pH levels. Elastin, however, possesses very few positively charged side chains in acid solutions and should not repel the charged orcein molecule.

In the foregoing discussion elastic tissue has been assumed to be equivalent to the characteristic protein elastin which it contains. It is possible, of course, that the staining of elastic tissue with any particular dye is due to other components. Elastin differs chemically from collagen in some important respects, chiefly in possessing far less

arginine, histidine and lysine and more leucine and valine. It contains no tryptophan but has slightly more tyrosine than collagen (Graham, Waitkoff and Hier, 1949). These differences might be expected to shift the isoelectric point from the acid towards the neutral point, which in fact they do. The isoelectric point of collagen is in the region of pH 4·85 and that of elastin about pH 6·0 (Bowes and Kenten, 1949). Leucine residues are known to be lipophilic (Davies and Dubos, 1947) and elastic tissue might, on this account, contain more lipid than collagen. Chemical assays, however, do not reveal any difference in the lipid content of the two substances. The literature concerning the action of proteolytic enzymes on elastic tissue is to some extent contradictory, nevertheless it appears that crude trypsin attacks elastic fibres only very slowly in their native state while crude pepsin acts more rapidly. According to Lansing (1951) the pure enzymes have no effect. Baló and Banga (1950) produced from pancreas a specific elastase which dissolves the elastic tissue of the aorta without hydrolysing it (see Chapter XXII, p. 625), and this enzyme has been shown by Pepler and Brandt (1954) to be identical with chondromucinase. Some authors (Braun-Falco, 1956 ; Harms, 1957) believe that the effect of elastase in tissues is to remove a chromotropic, mucopolysaccharide-containing cement substance, leaving the fibre (elastin) unaltered. Banga (1951) considered that the enzyme acted on elastin itself, converting it from a fibrous to a globular (soluble) form. During the process an oily layer rises to the top of the incubation medium, and this is said (Dempsey and Lansing, 1954) to have sphingomyelin-like properties.

Elastic fibres from all sources are remarkably insoluble in inorganic or organic solvents, differing in this respect from collagen which dissolves, for instance, in 2 per cent. acetic acid. This difference in reactivity may be connected with the fact that elastin contains 90 per cent. of non-polar amino-acids, and collagen only 50 per cent. Most studies on the property of elastic fibres have been made on material from the ligamentum nuchæ of the ox. Ordinary histological methods of staining suggest that there are wide differences between this material and the elastic fibres of skin, aorta and of the elastic laminæ of the smaller vessels. The elastic fibres of the human dermis are readily stained even with " bad " resorcin-fuchsin mixtures, while the finer fibrils of arterial walls are more difficult to stain satisfactorily. Such differences remain to be explained, but they are most probably of a physical nature.

Electron microscope studies of elastic tissue have not been altogether satisfactory. Gross (1949) observed that tryptic digestion of elastin from various sources released large numbers of filaments, polydisperse in length but constant in diameter. A large proportion of these

appeared to be tightly coiled helices composed of two intertwining threads. Franchi and de Robertis (1951), however, repeated the work of Gross and showed that the latter's findings were due to bacterial contamination of the trypsin used for digestion. Lansing *et al.* (1952) studied the process of digestion by elastase and noted the progressive splitting of large elastic fibres into small short ones. In thin sections fixed in buffered osmic acid elastic fibres appear (Dempsey and Lansing, 1954) as a negative image. Only in shadowed preparations can elastic tissue be resolved as an anastomosing, three-dimensional network of fibrils with an average diameter of 200 Å. Collagen fibrils surround the elastic fibres and occasionally penetrate them.

The observed histochemical reactions of elastic tissue are of interest, especially the positive pseudoplasmal reaction. This is due to the presence of oxidized phosphatides whose carbonyl (aldehyde) groups have been demonstrated in elastic tissue by means of the phenylhydrazine and dinitrophenylhydrazine reactions (Albert and Leblond, 1946 ; Schrek and McNamara, 1947) as well as by the hydroxynaphthoic acid reagent of Ashbel and Seligman (1949). When this last reaction is employed on formol-fixed frozen sections the internal elastic laminæ of arteries are invariably strongly stained ; elastic fibres in the skin, however, usually do not stain, although in both situations a pseudoplasmal reaction occurs. Both Sudan black and Baker's acid hæmatein technique (Chapter XI, p. 312) usually fail to reveal any phosphatide in elastic fibres, although chemical analyses reveal about 1 per cent. of lipid. Hack (1952), moreover, using freeze-dried carbowax-embedded material, has failed to demonstrate acetal phosphatides in elastic tissue. Interpretation of the positive copper phthalocyanin reaction (Chapter XI, p. 316) is difficult. Since nucleic acids and basic amino-acids are definitely absent I feel that it is due to the " sphingomyelin-like " component. Pyridine extraction leaves a positive reaction, as does extraction with acetone and ethanol. This is reversed, however, by boiling $CHCl_3/CH_3OH$.

Staining with thiazine dyes shows a strict and invariable orthochromasia, but the fibres, though isotropic by polarized light, possess a strong yellowish autofluorescence in ultraviolet light. The significance of these three characteristics is not absolutely clear, though isotropism presumably indicates a random orientation of molecules and autofluorescence may be due to lipid despite its presence in small amounts. It is worthy of note that the stretched fibres do exhibit birefringence, but this is of course never to be observed in histological preparations. The weak reaction obtained with various modifications of the Sakaguchi method for arginine reflect the low figures recorded for this amino-acid by chemical assays (Stein and Miller, 1938). With the tetrazonium reaction the colour of elastic tissue in the skin is a golden-brown (weak

positive), compared with the strong reddish-purple of collagen, while arterial elastic tissue is even paler. This finding might be due to the absence of histidine though one would have expected the increase in tyrosine, compared with that present in collagen, to have effectively balanced this. Tryptophan is absent from both collagen and elastin. The significance of the tetrazonium reaction here is obviously doubtful. Unless the reactive (tyrosine) groups are much more widely spaced than the reactive (tyrosine, histidine) groups of collagen it is difficult to explain the very pale colour produced in elastic fibres. There is no evidence to suggest that the tyrosine groups are in any way protected from reaction with tetrazonium compounds, in fact the opposite is the case. When the tetrazonium reaction is performed after benzoylation, whether heat has been applied to the sections or not, elastic fibres are always completely colourless. This indicates that their reactive groups are not so arranged as to be resistant to benzoylation, and they are unlikely to be so arranged as to fail to combine with tetrazonium salts, although this possibility still exists.

From histochemical evidence, therefore, we can tentatively suggest that elastic fibres owe their peculiar properties to (*a*) the relative absence of basic amino-acids in the molecule, (*b*) to the wide spacing of the various histochemically reactive amino-acid residues in the polypeptide chain, and (*c*) to the presence of oxidized phospholipids. In what way these factors operate remains a matter for speculation. No single reaction for SO_4 groups is positive in elastic fibres under histochemical conditions, and I cannot, therefore, agree with the views of Harms (1957) on the importance of ester sulphates as an explanation of the observed staining peculiarities of elastin. Moreover, even when the PAS reaction is positive, it has not been shown with certainty that this is due to carbohydrate. Differences between elastic fibres in various situations in the body are more likely to be due to physical than to chemical causes.

Pathological Changes in Elastic Fibres and Elastotic Degeneration. Elastic fibres are very stable components of the connective tissues and vessels, and (α-C^{14}) glycine studies indicate a very low turnover rate. With advancing age, however, marked changes occur. These take the form of longitudinal splitting, breaking into fragments and ultimately into granules. These changes are associated with chemical changes in amino-acid content (raised glutamic and aspartic acids) and in calcium content (up to 14 per cent.). The process of senile elastosis was studied by Weidmann (1931) and later by Cooper (1952) and by Lansing *et al.* (1953). Gillman and his associates (1954, 1955) have studied the so-called elastotic degeneration of collagen, and stress the value of Mallory's phosphotungstic acid-hæmatoxylin in distinguishing between normal collagen (reddish-orange) fibrinoid

(purple) and elastotic collagen (pale yellow or unstained). According to these authors elastotically degenerated fibres may arise (*a*) by degeneration of pre-existing collagen or (*b*) *de novo* by new formation of abnormal collagen.

Fibrin

Histologically, two staining methods in particular have been used to demonstrate fibrin. These are the methyl-violet method of Weigert (1887), usually performed as the Gram-Weigert method (Mallory, 1938) and the phosphotungstic acid-hæmatoxylin method (Mallory, 1938). The former requires a fine judgment in differentiation and, of course, stains many other structures, of which the majority are easily distinguished from fibrin. Collagen fibres react quite regularly, however, and these are not so easily distinguishable. The second method gives a deep blue colour with fibrin and although nuclei, muscle, neuroglia and coarse elastic fibres also stain blue, collagen and reticulin are red. Material which is not stained blue by phosphotungstic acid-hæmatoxylin is not fibrin. Fibrin is derived from the fibrinogen of the plasma which represents about 4 per cent. of the total plasma proteins. Fibrinogen is converted into fibrin by the addition of small quantities of thrombin, the amount of fibrin formed being independent of the amount of thrombin present, over a wide range (Morrison, 1947).

The amino-acid content of fibrin, in which the three basic amino-acids are strongly represented, accounts for the strong affinity for acid dyes observed when ordinary histological stains are employed. Human fibrinogen is a large elongated molecule with a length of 700 Å and a molecular weight of 400–700′000 (Edsall, Foster and Scheinberg, 1947) which undergoes no intramolecular rearrangement when changed into fibrin in the process of clotting (Bailey, Astbury and Rudall, 1943). The latter process has been described by Ferry and Morrison (1947), and by Ferry (1948), as the formation of a network whose fine strands consist of chains of fibrinogen molecules joined end to end and occasionally cross-linked by primary chemical bonds formed under the influence of thrombin. These fine strands are joined into coarse strands by secondary cross-linkages which form them into bundles. When the coarse clot is condensed into a film it becomes anisotropic (Ferry, 1948).

Proteolytic enzymes rapidly digest fibrin in unmodified condition and fairly rapidly even after treatment with formalin. Heat treatment, such as exposure to steam at 121° in the presence of 20 per cent. of moisture, increases the resistance of fibrin to enzymic digestion and increases its affinity for both acid and basic dyes (Ferry *et al.*, 1947). These changes, paradoxically at first sight, are attributed to secondary cross-linking (Ferry and Morrison, 1947). It appears that although

both heat and formalin prevent a certain number of the charged groups of the protein fibre from reacting with dyes, they increase the availability of all remaining charged groups by some physical modification of structure.

Jaques (1943) studied the reducing capacity of fibrinogen and fibrin for H_2O_2. He found that fibrinogen contained a reducing group with much stronger properties than tyrosine or tryptophan and that conversion to fibrin increased the number of these groups. Of naturally-occurring substances, only cysteine and ascorbic acid possessed a comparable reducing capacity weight for weight. The former is present in insufficient concentration to explain the findings, the latter is not present at all.

Histochemically fibrin possesses properties which correlate well with those determined by physical and chemical investigations. It is invariably orthochromatic and does not exhibit autofluorescence in ultraviolet light. Birefringence is seldom observed in an entire patch of fibrin in the tissues, but often the superficial layers are strongly anisotropic. The Sudan black test for formol-fixed lipid is sometimes positive, though more usually negative, and probably depends on the inclusion of lipid or lipoprotein during the process of clotting. Baker's acid hæmatein method (Chapter XI, p. 312), however, gives a pale blue colour with fibrin, suggesting the presence of phosphatides. The PAS reaction is usually positive, sometimes strongly so. Studies on a series of artificial clots produced from fibrinogen in different ways (Gitlin and Craig, 1957) suggest that included substances may be responsible for this reaction to some extent also. If their clots were formed from fibrinogen and thrombin alone, Gitlin and Craig observed negative reactions with the usual histological stains. If more than 1 g. per cent. of albumin was added, or glutathione at a concentration higher than 0·05 g. per cent., all the histological reactions became positive. Lorand (1950) and Mihályi (1950) both described urea-soluble and urea-insoluble forms of fibrin, and Gitlin and Craig related their positive staining fibrin to the urea-insoluble form, suggesting that some property resident in structural bonds or in cross-linkages was responsible for the observed differences in reactivity.

With the tetrazonium reaction, after heating and before and after benzoylation, both fine fibres and coarse masses of fibrin stain deep brownish-red, suggesting that fibrin exists in a more " condensed " form than do collagen and reticulin, for instance (see Figs. 53 and 54).

The DMAB-nitrite reaction of Adams (Chapter V, p. 93) is strongly positive with fibrin, which contains 3·5 per cent. of tryptophan, and this reagent is a useful one for demonstrating fibrin deposits in the tissues. In most cases fibrinoid gives an equally strong reaction so

that the differential diagnosis between the two substances is not possible by this means. Similarly the alkaline tetrazolium reaction (Chapter V, p. 114) is intensely positive in both fibrin and fibrinoid.

Summing up, therefore, one may say that the distinction between fibrin and the various other scleroproteins is usually easy. Protein material which is strongly acidophil when stained with acid dyes and blue with Mallory's phosphotungstic acid-hæmatoxylin, which is tetrazonium-positive after mild heat and benzoylation, is almost certainly fibrin. The other reactions are of interest but they are unnecessary for diagnostic purposes. It is necessary to bear in mind the possibility of other constituents of plasma being present as inclusions in a fibrin clot.

Fibrinoids

The term fibrinoid covers a number of different materials with diverse pathogenesis. After its introduction by Neumann (1880) the word was used to describe substances found in the walls of arteriosclerotic vessels which resembled fibrin in their tinctorial behaviour. It was also used to describe changes in the smaller vessels in malignant hypertension, periarteritis nodosa and thrombo-angiitis obliterans. Clark *et al.* (1936) regarded the arteriosclerotic variety as derived from some constituent or constituents of blood plasma and certainly not from necrotic collagen. Grosser (1925) applied the word to the fibrin-like substance deposited in various layers of the placenta. Klinge (1933) described the fibrinoid alteration of collagen in rheumatic fever as being due to hypersensitivity and maintained that a similar mechanism was responsible for its appearance in a number of disorders now considered collectively as collagen diseases.

The type of fibrinoid referred to in Table 10 is that found in areas of collagen necrosis in the rheumatic group of disorders. Its nature is of particular interest at the present time and many theories exist to explain it. Bahrmann (1937) regarded the fibrinoid degeneration of connective tissues as due to necrosis of collagen, while Klemperer *et al.* (1942) regarded the fibrinoid of disseminated lupus and diffuse scleroderma as a coagulation of ground substance. Altschuler and Angevine (1949) went further than this and, after performing a number of histochemical tests, they concluded that fibrinoid of the rheumatic variety was due to precipitation of the acid mucopolysaccharide of the ground substance by its combination with an alkaline (basic) protein. Glynn and Loewi (1952) also used a variety of histological and histochemical methods on rheumatic fibrinoid and considered that the change was essentially due to the deposition of a polysaccharide-containing substance in the altered collagen produced by the initial disorder. These last authors found that the positive PAS reaction of

rheumatic fibrinoid could be reversed by means of an enzyme described as pectinase (Chapter XXII, p. 619), but not by hyaluronidase, and they attributed the increased resistance of both fresh and fixed fibrinoid to the action of proteolytic enzymes as being due to protection by polysaccharide.

I am substantially in agreement with Glynn and Loewi's findings but, using a commercial sample of pectinase (Pectozyme, Evans and Rais, Ltd.), I did not succeed in reversing the positive PAS reaction of fibrinoid. Additional evidence derived from the tetrazonium reaction suggests that another change occurring in collagen on its conversion to fibrinoid is a " condensation " of the type experimentally producible in collagen by dry heat, which results in a change of colour from purple to deep brownish-red and to loss of birefringence. This last finding indicates a physical alteration in the axial alignment of the constituent collagen fibrils. If the positive PAS reaction indeed signifies the presence of polysaccharide, as seems likely in view of the absence of lipid substances indicated by biochemical and histochemical studies, there are at least three possible sources for the PAS reactive groups. As Glynn and Loewi suggest, they may be derived from external sources. Alternatively they may be derived either from the polysaccharide of the cement substance between the individual fibres, or from 1 : 2-glycol groups (see Chapter IX, p. 231) present in the collagen itself. In these last two cases it must be presumed that the reactive groups are normally not free to react, either on account of substitution (by sulphate, for instance) or because of protection by physico-chemical forces.

The results of X-ray diffraction and electron microscope studies (Kellgren *et al.*, 1951) indicate that fibrinoid of rheumatic origin contains no fibrin whatsoever. Material from those parts of a rheumatoid nodule containing large amounts of normal collagen on histological examination also show normal collagen fibres under the electron microscope, but give an X-ray diagram of disorientated collagen. In the parts showing advanced fibrinoid change the X-ray diagram of collagen is absent and electron microscopy shows only amorphous material. Intermediate areas between these two are common. Recent work on the chemistry of connective tissue fibres in rheumatic fever (Consden, Glynn and Stanier, 1952) has shown that the material in the central eosinophil core of the rheumatic lesion closely resembles collagen. By chromatographic methods these authors estimated the amounts of hydroxyproline, tryptophan, tyrosine and reducing sugar, in extracts of rheumatic nodules and the normal subcutaneous collagen of the elbow region. The nodules differed only in containing slightly more tyrosine and much more reducing sugar (2·9 as against 1·3 per cent.). Ziff *et al.* (1953) made alkali or trypsin extracts of five rheumatoid

nodules which they analysed for hydroxyproline and found 0·0–0·3 mg. per gram of tissue. The collagen content of the nodules, measured by the method of Lowry, Gilligan and Katersky (1941), was between 70 and 159 mg. per gram. Microscopical determination of the collagen/fibrinoid ratio in the nodules gave an estimated fibrinoid content of 17–40 mg. per gram. The maximum amount of collagen present in fibrinoid was estimated to be 2 per cent. by weight, that is to say, a negligible amount.

The nature of the fibrinoid of human placenta was studied particularly by Singer and Wislocki (1948). These authors carried out controlled staining in dilute solutions of orange G and methylene blue at various pH levels. They concluded that the staining characteristics of fibrin and this type of fibrinoid were essentially similar, and the same conclusion was reached by Busanny-Caspari (1952, 1957), who studied the morphogenesis of fibrinoid in placenta and decidua. Other types of fibrinoid have now been the object of critical histochemical studies. Montgomery and Muirhead (1957), using a broad spectrum of histochemical techniques, compared vascular fibrinoid with connective tissue fibrinoid and with fibrin and found the last two tinctorially and histochemically similar. In this respect their findings are in agreement with those of Gitlin, Craig and Janeway (1957). These authors, using the fluorescent antibody technique with rabbit anti-human fibrin, found that the fibrinoid of the collagen diseases (rheumatic arthritis, disseminated lupus erythematosus, dermatomyositis, rheumatic fever, etc.) was partly composed of fibrin. Moreover, they showed that conventional fibrin stains (Mallory-trichome and phosphotungstic acid hæmatoxylin, PAS) were often negative when the fluorescent technique gave a positive result. According to Montgomery and Muirhead (also Muirhead *et al.*, 1956) vascular fibrinoid differs from connective tissue fibrinoid in containing a quantity of cholesterol esters and potassium salts. They believe that the fibrinoid of renal cortical necrosis due to the Schwartzmann phenomenon may originate from smooth muscle and that vascular fibrinoid may have a similar origin. Fibrin and connective tissue fibrinoid gave a strong (Ehrlich-Lison) indole reaction whereas vascular fibrinoid did not. These results should be retested with the more sensitive indole reactions recently developed (see Chapter V, pp. 92–99).

Somewhat different conclusions were reached by Wolman and Laufer (1956), who concentrated largely on the protein make-up of fibrin and fibrinoid. Using the ninhydrin-Schiff reaction (p. 793) and a variety of blocking techniques, they showed that the fibrinoid of renal arteriosclerosis and of polyarteritis nodosa contained less protein (NH_2 groups) than fibrin. With the colloidal iron reaction (p. 256) all types of fibrinoid contained acid mucopolysaccharides, in contra-

distinction to fibrin, though an exception was recorded in the case of Nitabuch's layer and Rohr's striæ in the human placenta which, behaving otherwise as fibrin, were strongly Hale-positive. Since acetylation (p. 243) completely blocked the staining of all fibrins and fibrinoids with the PAS reaction and with Weigert's fibrin stain (p. 828), Wolman and Laufer believed that both are due to 1,2-glycol groups. They considered that the staining characteristics of fibrinoids and fibrin were due to the presence of polysaccharides and polyamino-sugars, present in different amounts.

It is clear that there are still outstanding differences of opinion on the question of the nature of the fibrinoids and that these are only partly due to real differences between the various materials studied by different authors. In particular, the difference between the results of standard histochemical methods and those obtained by the fluorescent antibody method are worthy of further study.

Keratin

It is necessary to consider, only briefly, the various properties of keratin which contribute to its histological distinction. This fibrous protein is characterized by its high content of the basic amino-acids arginine, lysine and histidine and of the sulphur-containing amino-acid cystine. Usually easily distinguished on morphological grounds alone, it has a strong affinity for both basic and acid dyes and is impervious to the action of trypsin and pepsin unless the disulphide bonds of cystine, which bind together the individual polypeptide chains of which the molecule is composed, are first broken by some means or other. Keratin in hair differs from that found elsewhere in its particularly high values for cystine, and in its correspondingly lower values for cysteine. Keratin in the stratum corneum and in the flaky contents of epidermoid cysts, as well as that in hair shafts, differs from prekeratin in the other layers of the skin in a physical manner also. In the first three situations it shows simple birefringence by polarized light (see Figs. 57 and 58). This property cannot be used to determine the presence of keratin, in malignant cells for instance, where it exists in isotropic form. The Sakaguchi reaction for arginine is not of use in distinguishing keratin from other materials with a smaller arginine content and the older lead acetate method for cystine only gives a reliable positive reaction in the case of hair shafts, whose identity is unlikely to be in doubt. In the identification of an unknown but morphologically suggestive material in the tissues as keratin, the property of resistance to trypsin and pepsin may be of some assistance. Prekeratin, however, with which we are most concerned, is much less resistant than hair and stratum corneum. The specific keratinase present in the intestine of the larval clothes moth, and active in a

reducing medium at pH 9·0, has not been purified for histochemical use.

There remain four methods which are of assistance in distinguishing between keratin and other acidophil, fibrous or globular, proteins of high arginine content. The first is the tetrazonium reaction after at least 18 hours benzoylation (with or without previous heating), which is uniformly negative both with keratin in hair and in the stratum corneum (Fig. 59) and also with epithelial pearls and with the intracellular form. The second method, which is sometimes useful, is the performic acid-Alcian blue technique (PFAAB), described in Chapter V (p. 111). This method is not a reliable indicator of the presence of prekeratin in tumours of squamous origin although it will invariably give a positive reaction at the point, in epithelial pearls for instance, where the diagnosis would be considered certain on histological grounds alone. The changes which occur during the production of keratin, described below, to some extent explain the failure of this reaction to demonstrate prekeratin. The third and fourth methods, the SH techniques of Bennett (1951) and of Barrnett and Seligman (1954) are considered after the discussion below.

The Process of Keratinization. One of the principal changes which takes place during cornification in normal human skin is the oxidation

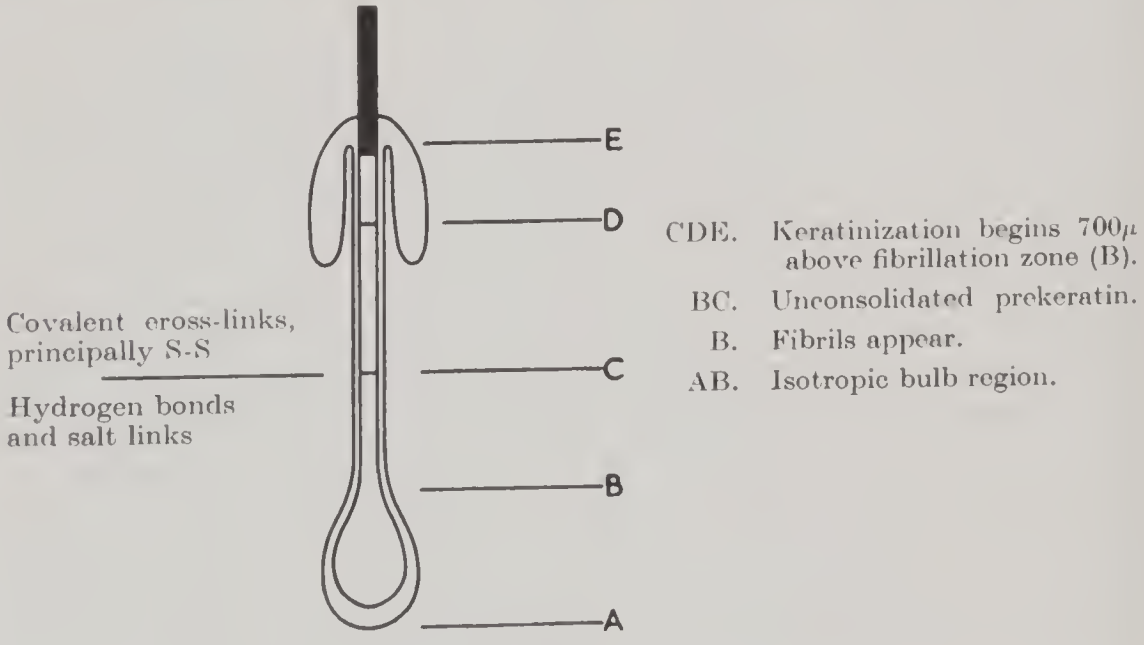


Fig. 60.

of SH groups in the lower strata to form SS groups in the stratum corneum. Wilkerson (1934) and Wilkerson and Tulane (1939) found that the keratinization of skin into nails and hair was accompanied by an increase of cystine and a decrease in methionine. The histidine, lysine and arginine ratios were the same in both cases. Bonting (1950) estimated cysteine, cystine and methionine in the skin of young and

adult rats. His findings agreed with those of Klauder and Brown (1936) that adult skin contained only 60 per cent. of the total sulphur present in skin from young animals. During maturation, that is to say during progress from infancy to the adult state, although the percentage of SS groups remained constant the total cystine decreased to 44 per cent. Bonting considered that the cystine lost from the young skin was transferred to the keratin of adult hair. Van Scott and Flesch (1954), however, have pointed out that significant amounts of SH occur in all horny structures and that the SS content of the Malpighian layer of the sole of the foot is the same as that of the stratum corneum.

Mercer (1949) studied the development of human hair by means of birefringence measurements and by X-ray diffraction as well as by histochemical tests. The diagram on page 176 (after Mercer, 1949) describes his main findings.

The hair cortex is divided into four regions. AB is the region of the isotropic bulb, BC the zone of fibrous but unconsolidated prekeratin, CD the zone of progressive hardening and DE the fully hardened zone. Mercer found that 0·1 per cent. trypsin at pH 8 and 40° for 2 hours removed the prekeratin of zone BC, but that the inner root sheath was more resistant and appeared to begin hardening at level B. The fibrils in zone BC were dispersed by a saturated solution of urea and disorientated by warming (90° for 30 seconds in water) with a corresponding fall in birefringence. In the whole prekeratinous zone BE, thiols were present in large quantities by the method of Giroud and Bulliard (see Chapter V, p. 100). Eisen, Montagna and Chase (1953) described the characteristic phases of hair-growth in the mouse and guinea-pig using the RSR and DDD methods for sulphydryl groups. The three phases are (1) *Anagen*, (2) *Catagen*, and (3) *Telogen*. During the first there is active cell proliferation, and during this period Bern (1954) has observed a high ^{35}S activity in the keratogenous zone 8 hours after the administration of radiocystine. During the second phase (catagen) proliferation ceases, the hair follicles become shorter and the hair club begins to form. The third phase (telogen) is the resting phase. Details of the distribution of SH and SS groups in human skin are given by Montagna *et al.* (1954) and the whole subject is treated by Montagna (1956) in his excellent book on the " Structure and Function of the Skin."

Eisen *et al.* observed that the results of the RSR and DDD reactions were equivalent, but that contrast was sharper with the latter. They found that during telogen SH was confined to the region around the hair club, but that in early anagen it appeared in the thickened epidermis. Later in this phase the greatest concentration of SH occurred in the cortex and cuticle of the keratogenous zone of the hair shaft (BD in Fig. 60), and this accords well with Bern's findings with

^{35}S. During catagen the brush region of the keratinizing hair club contained very large quantities of SH, and the amount in the external and internal hair sheaths was greater than in anagen. Neither Eisen *et al.* nor Van Scott and Flesch (1954) approve of the terms hard and soft keratin, but these are still widely employed to distinguish the alkali-resistant eosin-refractory and picric acid staining keratin, typically found in hair cortex and in the conical papillæ of the rodent tongue, from the soft eosinophilic keratin of squamous epithelia.

All these observations on the process of keratinization help to explain the failure of the PFAAB and PFAS reactions in the case of prekeratin. Fig. 36 (p. 97) shows that the second reaction begins to be strongly positive somewhere between levels C and D. This is the zone in which keratinization begins. It is apparent that for a positive PFAS reaction to occur not only a high content of cystine is necessary but also the physical state of orientation which gives the α-keratin X-ray diagram. Because most of the protein in squamous cell tumours is prekeratin rather than keratin, methods for SH are more likely to help than those for SS in establishing the keratinizing nature of a tumour whose cells contain acidophil protein. Bennett's mercurial method and the DDD method are the two most suitable in practice ; both are considered in Chapter V and in Appendix 5. There is little to choose between them in the matter of sensitivity, but in either case the investigation is complicated by the absolute necessity for controls.

Neurosecretory Substance (NSS)

It is now accepted that the hormones of the posterior lobe of the pituitary gland are produced in the hypothalamus and transported along the axons of the hypothalamo-hypophyseal tract. This hypothesis (Scharrer and Scharrer, 1937) was for a long time opposed to that of Gersh (1939), who held that the hormones were secreted by the pituicytes of the infundibular process. It was not until Bargmann (1949, 1950) successfully modified Gomori's (1939) chrome-hæmatoxylin stain (see Appendix 7, p. 819, and Fig. 61) and applied it to the demonstration of NSS that the Scharrer's hypothesis was confirmed.

Later Dawson (1953) showed that, after mild oxidation, Gomori's (1950) aldehyde fuchsin stain could also demonstrate NSS. Sloper (1955) used a modification of Matsmura's (1925) phosphotungstic acid-Congo red method, which stains the material blue. The mechanism by which these three methods stain NSS is poorly understood and they are in no way specific for the purpose. Chrome hæmatoxylin stains nuclei, lipofuchsin, Nissl substance and the anterior pituitary basophils. The other two stain these cells and also elastic tissue.

The oxytocic and anti-diuretic principles, long known to be present in extracts of the neurohypophysis, have now been shown by du

Vigneaud and his colleagues (Turner, Pierce and du Vigneaud, 1951 ; du Vigneaud *et al.*, 1953, 1954) to be two distinct octapeptides, both of which contain relatively large amounts of cystine. As a result of this work histochemical techniques for cystine have been successfully applied to the demonstration of NSS (Barnett, 1954 ; Sloper, 1954), and Adams and Sloper (1955) used the PFAAB method with very good results. This must be considered the histochemical reaction of choice ; but it may also be used purely as a staining reaction (Sloper and Adams, 1956).

The stainable NSS was considered by Schiebler (1951, 1952a and b) to be a complex consisting of the posterior lobe hormones and a glycolipoprotein, and by Hild and Zetler (1953) to be a bearer substance. Sloper (1955) and Howe and Pearse (1956), using the dog and the rat respectively, were unable to find either a lipid or a carbohydrate component of the NSS. Both considered it to be a protein or polypeptide containing a relatively large amount of cystine, and both found that it was digested by trypsin and by pepsin. Berezin *et al.* (1955) produced some very interesting results with tissue homogenates. They showed that homogenates of various toad tissues (*Bufo arenarum*, *marinus* and *ictericus*) would remove from formalin-fixed tissues (4 hours, 37°) nothing except NSS and the β-cell granules of the pancreas. An interpretation of these results is awaited. Although the antidiuretic hormone contains arginine as one of its eight amino-acids the Sakaguchi reaction (Chapter V, p. 119) fails to give a positive reaction with NSS as does the copper phthalocyanin reaction (Chapter XI, p. 316), which stains arginine-containing basic proteins as well as phospholipids and nucleic acids.

Since it is the " classical " method for the purpose, the chrome hæmatoxylin method will probably continue to be widely used. It must be emphasized that the standard procedure (as given for staining lipofuscins in Appendix 23, p. 926) does not stain NSS. Bargmann's modification given in the Appendix to this chapter must be employed (see Figs. 61 and 62).

Anterior Pituitary Gland Mucoid Cells

The mucoprotein-secreting (PAS +) cells of the adenohypophysis are called mucoid cells, since their contained granules are composed of material falling into this group of the conjugated proteins. The majority of mucoid cells belong to the basophil series of the older terminology, but the terms are not synonymous since there is an overlap into the chromophobes. Adams (1957), using his PFAAB reaction, followed by a PAS-Orange-G routine (Appendix 7, p. 820), has shown that the granules of the mucoid cells can be divided into two types on a firm histochemical basis. These two types of granule,

designated S and R, are illustrated in Plate IIb, p. 264. The former contains cystine in relatively high concentration and its carbohydrate moiety (that part which is responsible for PAS staining) is removed by treatment with performic acid. The second type of granule contains either no cystine or, at all events, an insufficient amount to give a positive PFAAB reaction. Its carbohydrate component withstands performic acid treatment and is therefore stainable by the PAS routine after such treatment.

Correlation with other terminologies is not easy since the various methods are not equally applicable to the pituitary gland of any one species. The two glands most often studied are those of man and the rat. The results of various staining methods and histochemical reactions, given in Table 12, are derived mainly from Adams (1957).

TABLE 12

Reactions of S and R-type Pituitary Mucoid Granules

Method	R granule	S granule	Vesicles (vesiculate mucoids)	α-granule
PFAAB–PAS–OG	Magenta ++	Blue ++	Magenta +	Orange
PFAAB	— or Pale Blue	Blue	—	—
PAS	Magenta +++	Magenta ++	Magenta ++	—
PFA–PAS	Magenta ++	— or Pink	— or Pink	—
PFA–AF–LG–OG (modified Halmi)	Green	Purple	Purple	Orange
CT	Brown ++	Brown +	—	Brown +
CT post-benzoylation (Pearse, 1952)	Brown (β) ++	(γ) —	—	—
Wilson-Ezrin	(β) Magenta +++	(δ) Purple	Purple	Orange

PFAAB = Performic acid–Alcian blue
OG = Orange G
AF–LG = Aldehyde Fuchsin–light green
CT = Coupled tetrazonium

Since the original method of Halmi (Appendix 7, p. 819) will not work in human glands correlation between this and Adams' method is possible only in the rat. However, since the latter method works poorly in the rat correlation becomes almost impossible. The β-granule of Pearse (1952) and of Wilson and Ezrin (1954) is distinguishable as an R-granule, while the δ-granule of these authors and Pearse's γ-granule are S-granules.

Adams and Pearse (1959) studied the distribution of the two types of mucoid granule in various pathological states and, although it is

impossible to correlate either type of granule with a specific hormone, it is probable that R-type cells belong to the category of thyrotrophs (Halmi, 1952 ; Purves and Griesbach, 1951), and S-type cells to the category of gonadotrophs. The S-type cells are almost certainly responsible for the production of the cystine-containing protein, isolated by Li *et al.* (1943) and Sayers *et al.* (1943), which was originally but erroneously thought to be ACTH.

Pancreatic α- and β-cell Granules

The α-granules of the pancreatic islets have been distinguished by means of specific silvering techniques which blacken them but not the β-granules. Of these the Gross-Schultze and Gross-Bielschowsky methods have been consistently employed. The α-granules can also be distinguished by methods like Bensley's aniline acid fuchsin-methyl green method for mitochondria, which stains them red and the β-granules green. Mallory's trichome stain produces red α-granules and blue β-granules, but good differentiation is very dependent on fixation. Recently Glenner and Lillie (1957) have shown that the α-granules in several species can be specifically stained by the post-coupled benzylidene reaction for indoles (Chapter V, p. 95), and they attribute this to their content of glucagon, which has a high tryptophan content according to Mohnike and Boser (1953). This method of demonstrating the α-granules is more satisfactory than any of the staining methods and I consider that it should be employed in place of them.

Several authors have noted the association of the α-cells with a strong non-specific esterase. This is particularly evident with the α-naphthyl and indoxyl esterase techniques (Chapter XVI, pp. 464 and 467), and these can therefore be used as functional indicators of the presence of α-cells in those species (man, rat, etc.) in which the reaction is positive.

For distinguishing the β-cell granules of the islets Gomori's chrome-hæmatoxylin phloxin method has been the most popular method. It stains the β-granules dark blue and the α-granules red. Gomori's aldehyde-fuchsin stain, without prior oxidation, has been used as an alternative in recent years. Bangle (1956) found that after peracetic acid, permanganate or bromine oxidation, an acidic substance was produced in the β-cells which would take up a number of basic dyes from solutions having a pH as low as 1·3. These included basic fuchsin, aldehyde-fuchsin, crystal violet and methyl violet 2B. Of these aldehyde-fuchsin was unique in staining the granules more or less specifically without previous oxidation. If aldehyde-fuchsin is to be used for staining pancreatic β-granules it should be more than 8 days old. If it is less mature than this it can be extracted from the granules

by treatment with acid alcohol ; the mature dye is resistant to such extraction.

The β-cells of the pancreatic islets in man and the rat possess a strong acid phosphatase (Goessner, 1957). The azo dye methods for this enzyme (Chapter XV, pp. 439 and 445) are especially convenient for this purpose and the method may be used as a functional indicator for the β-cells, in conjunction with an esterase technique for the α-granules.

This association of acid phosphatase with the production of insulin, a disulphide-linked peptide, has a parallel in the association of this enzyme with the formation of S—S containing proteins and peptides elsewhere. The acid phosphatase of hair follicles, and of the neurones of the supraoptic and paraventricular nuclei, can be cited as instances of this. The mechanism by which this enzyme participates in the formation of S—S linkages is unknown.

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CHAPTER VIII

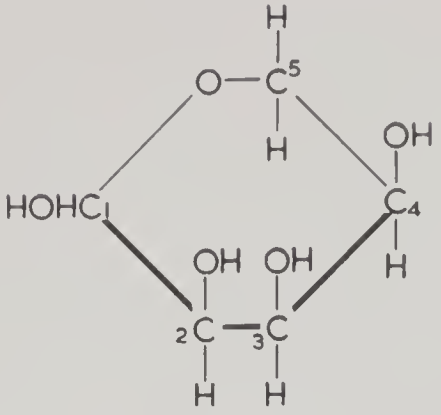
NUCLEIC ACIDS AND NUCLEOPROTEINS

Introduction

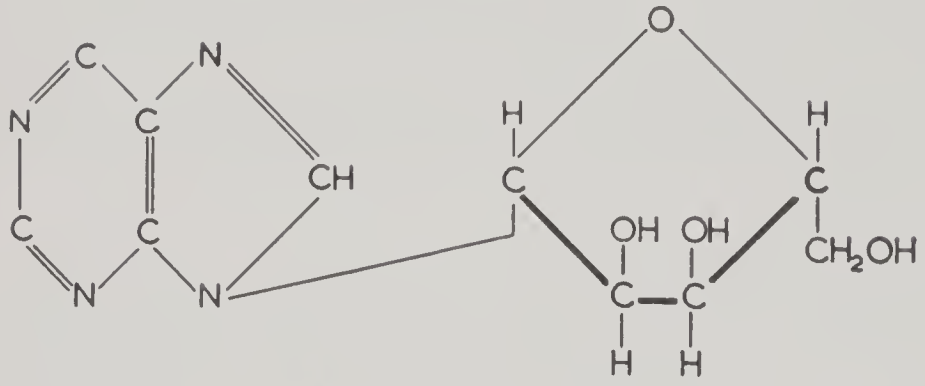
The first conjugated proteins with which we have to deal are the nucleoproteins, which are combinations of a basic protein with various highly polymerized polynucleotides (nucleic acids). Two types of nucleic acid are found in animal and plant tissues, the deoxypentosenucleic (deoxyribonucleic, thymonucleic) acid of the nuclei and the pentosenucleic (ribonucleic, plasmonucleic) acid of the cytoplasm, and they differ in many chemical and physical respects. However, both are precipitable from alkaline solutions by hydrochloric acid, and ribonucleic acid is also precipitated by acetic acid. Both form salts with alkaline earths or heavy metals and on hydrolysis they yield phosphoric acid, purine and pyrimidine bases, and a carbohydrate or carbohydrate derivative. Histochemical methods for their demonstration in the tissues have been based on reactions for all these constituents. According to the older, tetranucleotide, theory deoxyribonucleic acid (DNA) was regarded as a polymer made up of four distinct units (nucleotides) and containing a total of four PO_4 radicals, one residue each of two purine bases (adenine and guanine), one each of two pyrimidines (thymine and cytosine), and four molecules of the pentose sugar deoxyribofuranose.

New concepts, described below, have modified this theory so that it is now untenable. Chargaff (1955a) clearly states that the only recognizable sub-unit of structure in DNA is the mononucleotide. If repeating polynucleotide units recur in the nucleic acid chain there is, as yet, no way of demonstrating them. In spite of great differences in the nucleotide composition of nucleic acids certain features occur with regularity (Chargaff, 1950, 1951, 1955b). In all cases (1) The sum of the purine nucleotides is equal to that of the pyrimidine nucleotides. (2) The ratio of adenine to thymine equals 1. (3) The ratio of guanine to cytosine equals 1. (4) The number of 6-amino groups (adenine + cytosine + methylcytosine) equals that of 6-keto groups (guanine + thymine). Elson and Chargaff (1954) showed that this last feature, with uracil taking the place of thymine, applies also to some of the pentose nucleoproteins.

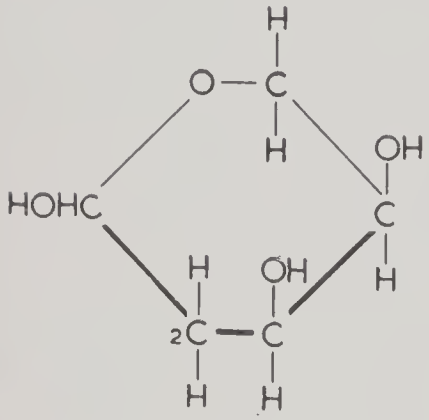
Some authors (e.g. Allen, 1954), besides dismissing the tetranucleotide theory, regard such terms as highly polymerized and depolymerized in respect of DNA as unjustified. The majority of authors,



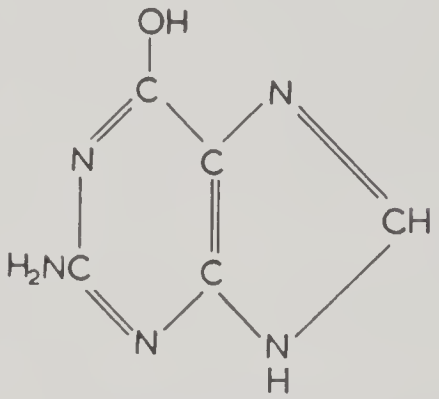
D-Ribose



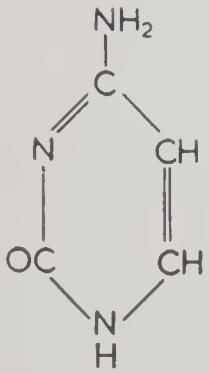
Adenosine (Nucleoside)
9-β-D-Ribofuranosidoadenine



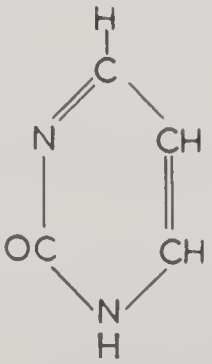
D-2-Deoxyribose



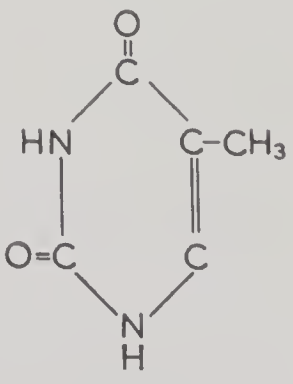
Guanine
2-amino-6-oxypurine



Cytosine
6-amino-2-oxypyrimidine



Uracil
2 : 6-dioxypyrimidine



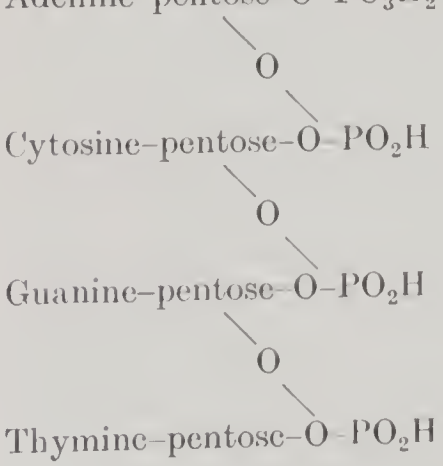
Thymine
5-methyl-uracil

however, continue to use them. Ribonucleic acid (yeast) differs from DNA only in yielding four molecules of ribofuranose in place of the deoxy sugar and one of uracil in place of the thymine of deoxyribonucleic acid. Since the identity of the pentose sugar has not been determined as ribose except in yeast nucleic acid, it is strictly more accurate to use the term pentose-nucleic acid instead of ribonucleic acid for the cytoplasmic variety, and deoxypentose nucleic acid for the nuclear variety, as far as animal tissues are concerned. Provided that this fact is recognized (Davidson, 1950), it is convenient and in conformity with common usage to employ the term ribonucleic acid (RNA) to indicate the pentose polynucleotide from any source. In this chapter, therefore, and elsewhere in this book, the term ribonucleic acid will be used although it is not strictly accurate. The formulæ for the two sugars and the five bases with which we are concerned are given on page 187.

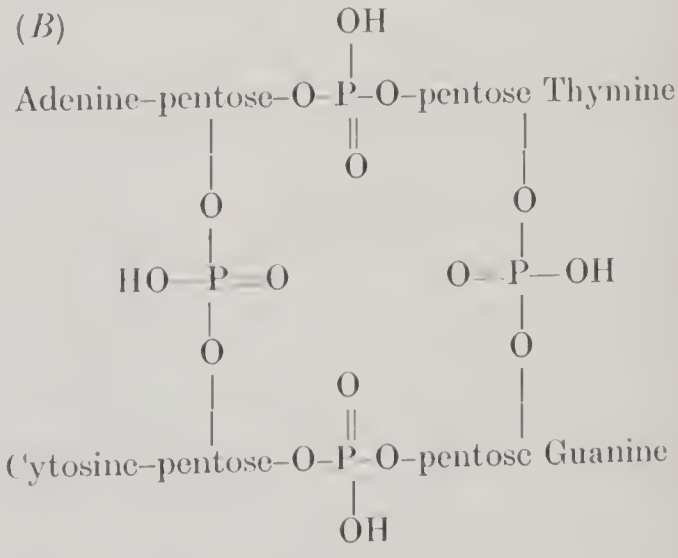
The Structure of DNA

Nucleosides are formed by the condensation of a pentose or a deoxypentose sugar with either a purine or a pyrimidine base, and nucleotides are the phosphoric esters of these. The nucleotides are the basal structures out of which the nucleic acids are built up, just as the amino-acids are built up into proteins. The older chemical evidence suggested that the nucleic acid molecule was a tetranucleotide containing one each of four different nucleotides. Two different structures were proposed for nucleic acids, the open chain structure of Levene and Sims (A), and the cyclic structure of Takahashi (B) :

(*A*)



(*B*)



Great advances in our knowledge of the structure of DNA have been made by a combination of chemical and physical studies. Using information derived from a number of sources Watson and Crick (1953) proposed the double helical chain structure for DNA which is illustrated

below (Fig. 63). The polynucleotide chains, which are coiled around a single axis, are held together by phosphoric acid diester bridges between the deoxyribose residues. The position of these bridges appears to be 3′, 5′ (Brown and Todd, 1955) in the case of calf thymus DNA, and the spiral is a right-handed one with the bases on the inside and the phosphates on the outside. The two chains are held together by the purine and pyrimidine bases, which are oriented perpendicular to the fibre axis. They are joined in pairs, a single base from one chain being hydrogen-bonded to a single base from the other chain. One of the pair must be a purine and the other a pyrimidine for bonding to occur. Wilkins *et al.* (1953) regard the Watson and Crick model as a simplification, which nevertheless fits most of the known facts. Their X-ray diffraction studies revealed no differences between samples of crystalline DNA from various species although the ratio of the bases varied widely. From these studies they deduced the helical structure of DNA and showed that a major part of the helix has one sharply defined diameter. Two coaxial helices of 18 Å diameter, one half pitch apart, surround a central helix of mean diameter 10 Å. For each turn of the helix there are 11 nucleotides whose shape resembles that of a rod inclined to the helical axis.

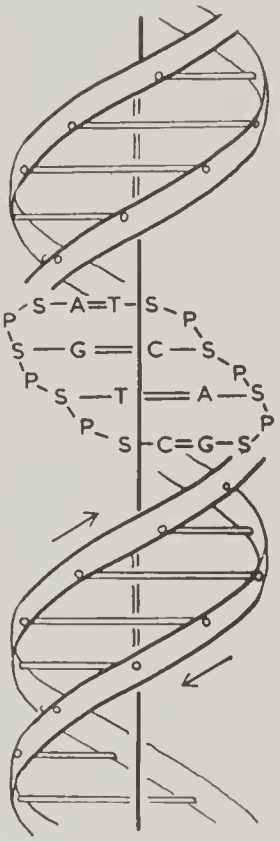


Fig. 63.

A symposium of authors (Feughelman *et al.*, 1955) agreed that the exact configuration of Watson and Crick was incorrect but found it possible to build a model containing the Watson and Crick H-bonding which gave the required Fourier transform.

These authors also considered the evidence for the combination of DNA with protein in the formation of nucleoproteins. In synthetic DNA-protamines the ratio between the numbers of basic amino-acids and phosphate groups may be varied but in sperm heads the ratio is always close to unity. This is assumed to indicate chemical combination. Protamine can be regarded as a polyarginine chain with repeating 4-arginine units. Nucleic acid presumably combines with poly-L-arginine by joining the four arginine guanidinium groups to the phosphate groups of the helix. For the combination of basic polypeptides with nucleic acids regular spacing of the phosphate groups is evidently necessary. Basic dyes (see below, p. 204) do not combine with mononucleotides; they are bound to nucleic acids not only by ionic bonds to PO_4 groups but also because they lie with their surface in close contact with the sugar surface of the nucleic acid molecule. It is

apparently quite feasible for short side chains to the main polynucleotide chain to occur as branches at triply esterified phosphate groups. These can lie in the grooves of the helix without interfering with the packing of the nucleic acid molecules and can terminate as secondary phosphate groups. These will provide additional binding sites for basic dyes.

Histochemically, the difference between the pyrimidines of the two nucleic acids is not detectable, but the difference between the two sugar residues is all-important. A third point of difference, the degree of polymerization of the constituent nucleic acid chains, is also of great histochemical importance. As stated in Chapter IV, the demonstration of this property is influenced by the type of fixative employed in the preparation of the tissues. This point has therefore to be considered in the interpretation of results, especially those obtained by the staining and extraction techniques described below.

General Reactions for Nucleic Acids

Reaction for Organic Phosphate (Serra and Queiroz Lopes, 1945)

Serra and his co-author demonstrated the phosphate radical in the DNA of the nucleus by hydrolysis and subsequent fixation of the released PO_4 groups with ammonium molybdate. The resulting phosphomolybdate was reduced to a blue compound with benzidine instead of the more usually employed stannous chloride. They employed either enzymic hydrolysis with nucleases or prolonged hydrolysis with N-HCl. The method is not very suitable for general use in histochemistry but serves to demonstrate the presence of PO_4 in the chromosomes. It may be objected that the prolonged hydrolysis, which these authors employed in the method finally evolved, would be likely to lead to artifacts due to diffusion of phosphate ions. This criticism is probably valid.

Reactions for Purines and Pyrimidines

The only reaction which has been used for the histochemical demonstration of purines and pyrimidines is the tetrazonium reaction after benzoylation or acetylation, already referred to at some length in Chapter V. It was first used in the histochemistry of nucleic acids by Mitchell (1942) for the demonstration of cytoplasmic ribonucleoprotein in tumour cells and subsequently by Danielli (1947) for staining chromatin in the chromosomes. Disregarding for the moment the other aspects of the tetrazonium reaction, it is clear that it stains particularly strongly nuclear chromatin, nucleoli, and such ribonu-

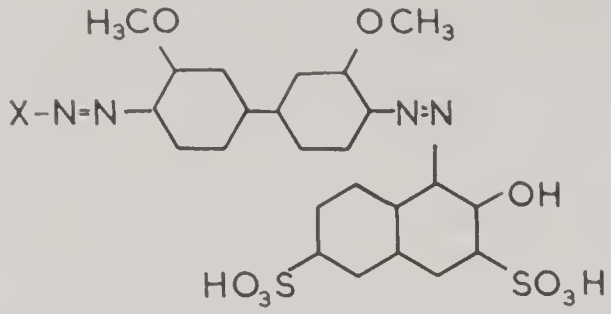
cleoprotein as is not removed during the process of benzoylation or acetylation. Only the interpretation of the reaction is open to question.

Both Mitchell and Danielli assumed that the development of a colour with tetrazotized benzidine, after the action of acetic anhydride or benzoyl chloride, was not due to histidine, tryptophan or tyrosine. They considered that it was most likely to be due to purine and pyrimidine groups, although Danielli felt that there might well be other substances in the tissues which would react with diazonium and bis-diazonium salts. Mitchell (1942) stated that adenine, guanine and uracil could react with diazonium salts *in vitro* and Danielli claimed that both DNA and RNA were able to react *in vitro* with tetrazotized benzidine. My own results (reported in the first edition of this book) with purified DNA-protein and with DNA almost free from protein, deposited in crystals of sodium chloride and fixed on slides with methanol, showed that any positive staining by the coupled tetrazonium reaction was due to the presence of protein since the pure DNA crystals completely failed to react. In view of this evidence it was stated categorically that the staining of nuclei by the tetrazonium reaction is not due to the purines and pyrimidines of their constituent nucleic acids. The positive reaction obtained by Burstone (1955) with DNA was almost certainly due to contamination with protein.

An attempt was therefore made to determine the nature of tetrazonium staining of the nuclei, before and after benzoylation, by further experiment. The application of mild heat (see Chapter V, p. 129) is not necessary to develop the resistance of nuclear chromatin to benzoylation, though if such heat is used the degree of resistance is increased, just as it is in certain simple protein components of the tissues. It seemed, therefore, probable that the reaction of chromatin after benzoylation was due to tyrosine, tryptophan or histidine groups, protected from benzoylation by the physical state of the whole nucleoprotein. This belief was supported by the fact that if the nuclei were subjected to extractions of the type described below in this chapter, and also in Chapter XXII, p. 601, which remove all nucleic acids, the protein structure which remained gave a positive tetrazonium reaction and therefore presumably contained one or other of the amino-acids.

Barnard and Danielli (1956) have now made a comprehensive study of the coupled tetrazonium reaction in relation to nucleic acids and claim that the reaction is specific for " protein in a special form of combination with nucleic acid." Observing that benzoylation *should* block the tetrazonium staining of all tissue components they endeavoured by fractionation studies to determine the cellular dis-

tribution of the reactant responsible for its failure to do so, that is to say of the protein-bound group X in the structure

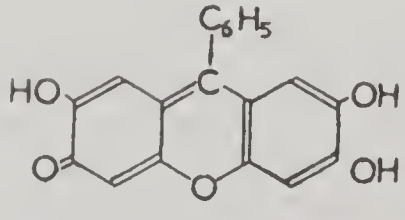


For this purpose they used chick erythrocyte nuclei coupled with Fast blue B salt (tetrazotized diorthoanisidine) and then with 2-naphthol-3,6-disulphonic acid to confer water solubility. Solvent extraction and trichloroacetic acid extraction showed that neither the lipid nor the nucleic acid fractions contained any of the dye component. Extraction of the residue with alkali yielded an intensely coloured macromolecular compound which gave a typical amino-acid mixture after reduction of the dye and hydrolysis. This process was achieved, not without difficulty, by refluxing in 0·015M-cetyltrimethyl ammonium bromide in 0·3 N-NaOH. Paper chromatography of the dye fraction in two solvents gave one main spot suspected to be aminohistidine. Barnard and Danielli suggest that a positive coupled tetrazonium reaction after benzoylation is normally limited to histidine residues in a nucleoprotein fraction and that failure to benzoylate histidine in tissue sections is due to " protective bonds " which exist only after dehydration.

It is now clear that the reaction cannot be used for the identification of nucleic acids but that it remains useful for demonstrating histidine-protection not only in nucleoproteins but in many other types of protein.

Reactions for Deoxyribose and Ribose (Turchini)

Turchini *et al.* (1944) developed a method for DNA and RNA depending on mild acid hydrolysis followed by reaction with phenyl (or methyl) trihydroxyfluorone. The formula for the phenyl compound is given below :



9-phenyl-2 : 3 : 7-trihydroxyfluorone

Although the method has the advantage that distinct and different colours are given by the two nucleic acids in plant tissues, for which the method was primarily evolved, the results in animal tissues are less satisfactory. Using the phenyl compound and the times of hydrolysis advised by Turchini *et al.*, the best that I could achieve in human tissues, fixed in a variety of alcoholic and formalin-containing fixatives, was a pale rose pink colour in the nuclei, nowhere approaching the clarity of definition given by the Feulgen reaction, described below. Backler and Alexander (1952), however, have reported the successful use of a modification of the Turchini reaction for the differential staining of DNA and RNA. Using the methyl substituted fluorone, DNA stained violet to blue black and RNA yellow to red. With this modification violet-stained nuclei can be obtained regularly but the results are dependent on the fixative employed and, in general, the times of hydrolysis and subsequent staining are longer than usually suggested.

Reactions Specific for Deoxyribonucleic Acid

The Feulgen (Feulgen-Schiff) Reaction

The reaction was introduced by Feulgen and Rossenbeck (1924) as a specific test for thymonucleic acid (DNA). It depends on the treatment of fixed tissues by mild acid hydrolysis (with N-HCl at 60°) which Feulgen showed could release aldehyde groups from the deoxypentose sugar of DNA. Following hydrolysis the tissues are washed and transferred to a solution of Schiff's reagent (fuchsin-sulphurous acid) which reacts with the exposed aldehyde groups to produce a purple dye in the nuclear chromatin alone (Fig. 64). Bauer (1932) worked out the optimum times of acid hydrolysis, which depend on the fixative employed, and his investigations were extended to plant tissue by Boas and Biechele (1932) and Hillary (1939). A chemical explanation of the reaction was produced by Stacey *et al.* (1946). These authors claimed that a relatively gentle acid hydrolysis would transform deoxyribose into ω-hydroxylævulinic aldehyde ($HOCH_2.CO.CH_2.CH_2.CHO$), and they suggested that this labile aldehyde was responsible for the purple colour given with Schiff's reagent in the Feulgen test. In consequence of the explanation offered by Stacey *et al.*, the specificity of the test, or more strictly speaking its specificity for the localization of DNA, became the object of increasing doubt. In a later section, the modern history of the Feulgen reaction is discussed. The arguments for and against it are put forward at some length and they serve to illustrate the complexity of the problem. For those who are interested solely in applying the reaction, it may be well to state here that its specificity for DNA is able to survive the conflict reported below, and that it may be used with confidence for the purpose.

Some Important Results of the Feulgen Reaction. Use of the Feulgen reaction for DNA has resulted in a great increase in our knowledge of the distribution of this substance in various types of cell. Darlington (1942, 1947) studied particularly the chromosomes and pointed out the vital importance of the reaction in making chemical processes in the nuclei structurally visible. Stowell (1942, 1945) and Stowell and Cooper (1945) used a modified Feulgen reaction, together with a system of photometric recording, to estimate the amount of DNA in a variety of normal and neoplastic tissues. By means of Stowell's method it is possible to measure mean amounts of substances per unit volume and per cell and thus to correlate quantitative results with morphological data. Further knowledge of the function of the nucleic acids in nuclear division has come from the studies of Thorell and Caspersson (1941), Thorell (1944), and Hydén (1943). All these authors used ultraviolet absorption techniques as well as the Feulgen reaction and from their work developed the concept of nucleolar associated chromatin. Caspersson (1947) considered that this material was responsible for the change in malignant cells which gives rise to the production of large quantities of protein. Davidson and Waymouth (1946) showed that in the nucleus of the rat liver cell the peripheral part of the nucleolus contained DNA and the central part RNA. Important information on the constancy of nuclear DNA has been derived from the studies of Vendrely and Vendrely (1948), Ris and Mirsky (1949), Pasteels and Lison (1950), and Swift (1950). The procedure used by the last author has been employed by a substantial number of workers in this field. Correlated chemical and quantitative cytochemical studies have been carried out in many cases but the problem of DNA in heterogeneous cell populations can be dealt with *only* by cytochemical means. Ris and Mirsky, for instance, showed that rat liver contained three classes of nuclei in which the DNA content (intensity of the Feulgen reaction) was close to 1:2:4. The polyploidy thus demonstrated was shown in other tissues by Pasteels and Lison and by Swift. These studies are further considered, from the cytophotometric angle, in Chapter XXVIII.

From the point of view of the histologist the Feulgen reaction is less useful, in practice, than theoretical considerations might indicate. It is often necessary to apply it solely in order to be able to state with confidence that a given basophilic inclusion contains no DNA. For this purpose it is the only convenient and acceptable reaction. It may also be found useful for nuclear sex studies if, for any reason, the use of ribonuclease or acid extraction for RNA is not feasible.

The Specificity of the Feulgen Reaction. Most of the doubts concerning the specificity of the Feulgen reaction were raised, and later sustained, by the researches of Stedman and Stedman (1943a and b,

1944, 1947, 1950). Other workers, including Serra (1943), Choudhuri (1943) and Carr (1945), also offered evidence against the specificity of the reaction. Stedman and Stedman (1943a) isolated an acidic protein containing 9 per cent. arginine, 5 per cent. histidine, 11 per cent. lysine and a considerable amount of tryptophan, from various nuclear sources. The whole protein was acidic, despite its considerable content of the basic amino-acids, because of its very large content of glutamic acid. This protein, which they called chromosomin, was originally regarded by its discoverers as the principal component of the chromosomes chiefly on account of its staining properties with methylene blue and with the hæmatoxylins. With the latter dye it assumed the ' typical blue colour familiar to histologists.'' Stedman and Stedman denied that the Feulgen reaction could localize DNA because they found that the red dye, produced by the addition of fuchsin sulphurous acid to the acid hydrolysis product of DNA, was soluble in water and could be taken up from aqueous solution by chromosomin. Choudhuri (1943) stained chromosomes in plant cells with the red dye produced as above, which he termed " developed nucleal stain.'' The Stedmans argued, therefore, that this red dye acted as an ordinary basic stain, that it was diffusible in the tissues, and that it stained the chromosomes by attachment to the acidic groups of chromosomin. It would thus be sufficient for DNA to be present anywhere in the nucleus, and not necessarily in the chromosomes, for a positive Feulgen test to occur. Callan (1943) suggested that the Stedmans' view could be refuted by the experiments of Mazia and Jaeger (1939) who used nuclease to reverse the positive Feulgen reaction in Drosophila salivary gland chromosomes, and by the ultraviolet absorption experiments of Caspersson (1936), who found the spectra of purines and pyrimidines in the chromosomes. He also cited the micro-incineration experiments of Norberg (1943), who showed that these structures contained nearly all the phosphate in the nucleus. Stedman and Stedman (1943b), however, considered that Mazia and Jaeger's results confirmed their own point of view in that deoxyribonuclease, while destroying the staining capacity of the chromosomes towards aceto-carmine, did so without affecting their integrity. The reason why the basophilia of chromosomin was apparently reversed by treatment with nuclease was not explained by these authors. The reversal of Feulgen staining brought about by deoxyribonuclease might be taken to support rather than refute the argument of Stedman and Stedman, since, in the absence of DNA, no soluble aldehyde-leucofuchsin will be produced. Dobson (1946) and Brachet (1947), however, showed that pure DNA, fixed in Zenker and embedded in agar, gave a strong Feulgen reaction.

The exchange of views continued with Caspersson (1944) and Stedman and Stedman (1944) presenting their respective arguments

on adjacent pages in the same journal. Throughout these often confusing arguments the real point at issue remained the same, the solubility or insolubility of the red reaction product produced in the Feulgen test. Baker and Sanders (1946) argued that if the aldehyde formed from DNA by acid hydrolysis could diffuse in the tissues it would also diffuse into the surrounding fluid, and Danielli (1947) criticized the explanation of the Feulgen reaction given by Stacey *et al.* on the grounds that a substance of such small molecular weight as ω-hydroxylævulinic aldehyde would certainly be washed out of the tissues during the reaction. Carr (1945) attacked the specificity of the reaction on three grounds. First, he suggested that chromosomes could regenerate the colour of Schiff's reagent by simple absorption in the manner of a column of alumina, and secondly, that selectivity for the nucleus depended on the destruction of cytoplasmic components, which would otherwise react, by the acid hydrolysis. Thirdly, he suggested that excess of SO_2 did not block the reaction with Schiff's solution as it should do if this was due to the formation of aldehyde groups. Stowell (1946a) reviewed the evidence for and against the specificity of the Feulgen reaction and concluded that it was relatively specific when properly controlled. Both this author and Dobson (1946) took pains to refute the experimental evidence of Carr, Dobson suggesting that Carr's first point was answered by the results, quoted above, which were obtained with nucleases. Stowell obtained far less satisfactory results with nuclease and did not draw any conclusions from its use. Carr's second point was answered more satisfactorily by Dobson, who proved, by weighing, that acid hydrolysis caused an insignificant loss of material from fixed tissue. The third point remained unanswered by Dobson or by Stowell, although Feulgen (1927) had shown that no colour developed with Schiff's reagent if the aldehyde groups responsible for the reaction were blocked with ammoniacal silver solutions or with bromine, and Brachet (1947) later showed that sodium bisulphite in a non-acid medium had the same effect. I have observed that dimedone or hydroxylamine hydrochloride, both of which combine with aldehydes to form unreactive compounds, will also block the reaction of hydrolysed nuclei with Schiff's reagent.

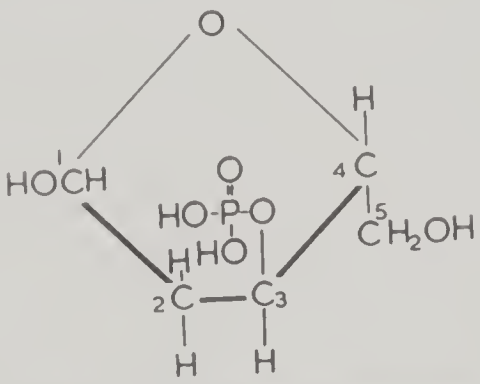
After these further exchanges between the orthodox school and their opponents, the point at issue still remained the solubility or insolubility of the Feulgen reaction product. More modern work throws light upon the still unsettled problem. Sibatani (1950) described a remarkable and at present unexplained effect of solutions of histones, or other proteins, in enhancing the colour developed in the Feulgen reaction with extracted DNA. The stage at which they were added did not influence the effect. He believed, therefore, that the dye produced in the reaction was diffusible and that its absorption

was not confined solely to chromosomin, as postulated by the Stedmans, but occurred with other proteins as well. Di Stefano (1948) studied the effect of Feulgen hydrolysis on the composition of cell nuclei fixed in acetic alcohol, by means of photometric measurements. He concluded that after 12 minutes' hydrolysis only the two purine groups were lost, while a further 12 minutes' hydrolysis removed all the phosphorus and histone of the nuclei. Stedman and Stedman (1950), however, by direct chemical estimations of isolated nuclei fixed in acetic alcohol, found that 10 minutes' hydrolysis removed all the histone and 27 per cent. of the phosphorus. These same authors also found that the hydrolysis fluid, separated from nuclei, gave an intense positive reaction with Schiff's solution. They concluded that since the form of the chromosomes was unaltered by a 10 minute hydrolysis, histones could not be present therein and, therefore, that nucleic acid combined as nucleohistone must necessarily be extra-chromosomal. These conclusions do not seem to follow from their experimental results although the latter do indicate that the structural form of the chromosome can be maintained in the absence of nucleic acids and histones. Kaufmann *et al.* (1948, 1950), as the result of experiments with enzymic and other extraction techniques on fixed material in tissue sections, concluded that chromosomes represented an integrated fabric in which no single protein or nucleic acid could be regarded as the primary structural component.

Recent experiments carried out by Kasten (1956) suggest that chromosomin plays no part in the development of a positive Feulgen reaction. This author prepared a modified Schiff's reagent by bubbling SO_2 into 1 per cent aqueous acid fuchsin. This dye is a mixture of trisulphonated triphenylmethanes and these could hardly be expected to combine with an acidic protein like chromosomin. In practice, Kasten was able to obtain Feulgen-type staining in hydrolysed nuclei with his acid fuchsin reagent and this result was not achieved in unhydrolysed tissues. The possibility that the reconstituted acid dye might be staining the basic (histone) protein of the nuclei has to be considered. The negative result obtained after various extraction procedures for DNA renders this explanation unlikely and we must conclude that Kasten's work provides further evidence against the chromosomin hypothesis. There is evidence, however, from the work of Chayen and Norris (1953) that *false* localization of DNA may be produced by acid hydrolysis. These authors found, in the root meristem cells of *Vicia faba*, some cytoplasmic particles removable by DNase but not by RNase. These were not stained *in situ* by the Feulgen technique but their DNA was shown to be transferred to the nucleus.

Further Work on the Mechanism of the Feulgen Reaction. The original explanation offered by Stacey *et al.* (1946) was generally

accepted, and its acceptance was responsible for much of the now historical argument given in the preceding section. Later, however, Overend and Stacey (1949) followed up the original work by studying the reaction of a large number of compounds in the Feulgen test. As a result, they explained the latter as follows : acid hydrolysis first breaks the sugar linkages engaged in polymeric bonding (Reaction I) and secondly ruptures the glycoside linkages between the sugars and the purine bases (Reaction II). The deoxyribose compounds thus revealed are described as being attached through PO_4 linkages in the main nucleic acid chain where they are firmly held in the furanose form, with exposed aldehyde residues. It is doubtful if they are as firmly attached as these authors suggest. According to Overend and Stacey only a few of the aldehyde residues are needed to give the maximum formation of dye with Schiff's solution. The whole theory explains the Feulgen reaction in a satisfactory manner. Reaction I can be considered as taking place in a simple tetranucleotide such as that shown on p. 188 (Formula A), although it is by no means certain that the deoxyribonucleic acids of the nucleus are present in this form. In Reaction II the arrangement at carbon atom 1 of the furanose sugar (illustrated below), after removal of the base (adenine), is that of a potential aldehyde. This group (HO.CHO) reacts as an aldehyde with Schiff's and other reagents. The ribose sugars, with an OH at carbon atom 2 in place of the second hydrogen atom of the deoxy sugars (see formula for D-ribose, p. 187), are not hydrolysed by normal HCl and therefore do not react in the Feulgen test. The exact mode of attachment of the fuchsin-sulphurous acid molecule to the sugar aldehydes has not been ascertained (see Chapter XII, p. 334). Probably two potential sugar aldehydes react with one molecule of fuchsin-sulphurous acid to give the red product on which the test depends.



2-Deoxyribofuranose-3-phosphate

Using Fisher-Hirschfelder atomic models based on van der Waals volumes Lessler (1951, 1953) found that the S to S distance in fuchsin-sulphurous acid lies between 10 and 11 Å. X-ray diffraction studies

indicate that the alternate nucleotides of DNA are about 10·2 Å apart. If indeed one molecule of fuchsin-sulphurous acid condenses with two molecules of aldehyde these must be situated on alternate nucleotides.

Li and Stacey (1949) showed that the loss of intensity of the Feulgen reaction by over-hydrolysis could be attributed to the lability of sugar aldehyde attachments and to instability of the furanose form itself. Overend and Stacey concluded that the Feulgen reaction located DNA at the site in which it occurred, proving this point by treating protein-free sperm DNA by hydrolysis in citrate buffer (at pH 3·0 at 100° for 2·75 minutes), removing the liberated purines by washing, and finding that the residual material gave an instantaneous reaction with Schiff's solution while the supernatant remained colourless for over 24 hours.

Lessler (1951) applied the Feulgen technique, under strictly controlled conditions, to DNA preparations embedded in gelatin. He concluded that the intensity of colour developed was proportional to the temperature and duration of hydrolysis and, within limits, to the concentration of DNA. By means of a variety of aldehyde-blocking reagents (see Chapter XII), applied after hydrolysis, Lessler was able to prevent subsequent coupling with Schiff's reagent. He therefore considered that the Feulgen reaction depended on the formation of aldehyde groups. Lhotka and Davenport (1951), however, conducted a number of experiments designed to elucidate the nature of the Schiff-positive radical responsible for the Feulgen reaction, and expressed doubts as to the validity of the usually accepted (aldehyde) mechanism. They attempted to produce a histological picture comparable with that obtained in the Feulgen reaction by using various reagents in place of fuchsin-sulphurous acid. These included ammoniacal silver nitrate (cf. technique for glycogen, Chapter IX, p. 268), phenyl hydrazine, 2-4 dinitrophenylhydrazine and *p*-nitrophenylhydrazine (cf. techniques for ketosteroids, Chapter XII, p. 355). In each case the results were negative. However, these authors were able to block the subsequent reaction of hydrolysed nuclei with Schiff's reagent, by two processes. First, they treated blocks of tissue, fixed in picrosulphosalicylic acid solution (see below), with 50 per cent. KOH in 50 per cent. alcohol in the presence of formaldehyde. After washing, the blocks were treated with Schiff's reagent and the usual sulphite baths but no colour was produced in the nuclei. This Cannizzaro reaction proceeds as follows:

$$2R.CHO + KOH \rightarrow R.CH_2OH + R.CO_2K$$

If the blocks were then rehydrolysed with the acid fixative they once more gave a positive Feulgen reaction in the nuclei. In the second process hydrolysed blocks of tissue were treated for 2–4 hours under

a reflux condenser with isopropyl alcohol containing excess aluminium *iso*propoxide, producing a reaction such as that illustrated below :

$$R.CHO + \underset{\textit{Iso}\text{propyl alcohol}}{CH_3CHOHCH_3} \xrightarrow{Al(OC_3H_7)_3} RCH_2OH + CH_3COCH_3$$

After washing, treatment with Schiff's reagent did not produce a Feulgen reaction. Lhotka and Davenport concluded that failure to demonstrate the presence of aldehydes with such specific tests as the oxidative (ammoniacal silver) and amine (hydrazine) reactions raised doubts as to whether an aldehyde was in fact responsible for the Feulgen reaction, despite their success in blocking the latter by other means. They considered that the reacting radical might be a ketone rather than an aldehyde, since many ketones would give a positive reaction with Schiff's solution, but that their failure to demonstrate an amine-carbonyl union weakened this premise. This work raises certain points for comment. First, as mentioned above, Feulgen (1927) himself used ammoniacal silver nitrate to block his reaction after acid hydrolysis of the nuclei, although he did not produce a histologically appreciable result by this means. Secondly, the various hydrazine compounds which Lhotka and Davenport used will also block the reaction and only fail to produce a histological result because the aldehyde-hydrazine compounds are colourless. If an aryl hydrazide is used followed by diazotized *o*-dianisidine as in the Feulgen-naphthoic acid reaction (see p. 203 below), a perfectly satisfactory result is obtained. Thirdly, as explained by Overend and Stacey, the reactive group in hydrolysed DNA is a *potential* aldehyde which may, therefore, not react equally well with all aldehyde reagents under the usual conditions of experiment.

From all the foregoing work it is clear that acid hydrolysis of nuclei causes two things to occur almost simultaneously. First, the purine bases are rapidly and completely removed and potential aldehyde groups are uncovered in the deoxyribose groups which remain : secondly, the histones and nucleic acids are progressively removed. Thus the Feulgen reaction is always a compromise between these two actions. After short hydrolysis the first predominates and application of Schiff's reagent at this point results in maximum staining of the chromosomes. As hydrolysis proceeds the second action gains momentum and the Schiff reaction increases in the hydrolysis fluid, while it decreases in the chromosomes. Finally, the second overwhelms the first and the chromosomes cease to react. It is significant that the optimum time of hydrolysis for the material used in di Stefano's and the Stedmans' (1950) experiments is in the region of 6 minutes.

The fact that over-hydrolysis produces a negative Feulgen reaction has been known since its earliest days.

The Feulgen Reaction in Practice. With ordinary histological materials the Feulgen reaction can be applied after almost any fixative except Bouin, with which excessive hydrolysis occurs during fixation. A list of the optimum times for hydrolysis with various fixatives, determined by Bauer (1932), is given in Appendix 8, p. 823. For ordinary work the modified Schiff's solution of de Tomasi (1936) has been widely used and for small materials, such as smear preparations, that of Rafalko (1946). A similar preparation, made by bubbling SO_2 gas through a solution of basic fuchsin, was employed by Itikawa and Ogura (1954). This has already found considerable favour with workers using Schiff's reagent for routine purposes on account of its ease of production and good stability. Coleman (1938) introduced the use of activated charcoal to remove residual colour in Schiff's reagent prepared by de Tomasi's method and this is a worthwhile modification. Lison (1936) advised the addition of 0·2 ml. of acetaldehyde per 100 ml. to the prepared reagent, standing for 30 minutes and finally adding a further 20 ml. of N-HCl and 1 g. of sodium sulphite. This modification has no advantages over the simpler method of Coleman, using activated charcoal. Lhotka and Davenport (1947, 1949a) applied a modified Feulgen technique to the staining of tissues in the block. Thin slices of tissue were fixed in equal parts of 5 per cent. sulphosalicylic acid and saturated aqueous picric acid for 48 hours, they were then washed and treated with Schiff's reagent for 24–48 hours. Structures other than nuclei, including myelin, elastic fibrils and cartilage, were also stained red by this procedure. Lhotka and Davenport (1949b) investigated the deterioration of Schiff's reagent under varying conditions. They found that two factors were involved, first an irreversible colour change caused by exposure to air and secondly, a partly reversible change caused by a shifting of the chemical equilibrium between sulphite and dye (Karrer, 1950). They concluded that Schiff's reagent could be prepared in large quantities and that, provided it was stored between 1° and 5° in tightly stoppered bottles, it would retain its staining efficiency for 6 months or longer. It is preferable, I think, to make up sufficient reagent for one month's use only, and to store it under the conditions advised by Lhotka and Davenport. In this way, if it is kept in the dark and used in the dark in a sealed container, deterioration is minimal and false reactions due to this factor are avoided. Alexander *et al.* (1950) gave details of a rapid method of preparing Schiff's reagent involving the simultaneous addition to the basic fuchsin solution of sodium hydrosulphite ($Na_2S_2O_4$, dithionite) and activated charcoal. Thorough shaking decolorizes the fuchsin in the absence of the usual

acid (20 per cent. HCl) but, since excess SO_2 is not present, oxidation must be prevented by layering with xylene. Sections were subjected to hydrolysis in the usual way and placed in the reagent for 2 hours.

A rapid method for the preparation of Schiff's solution was also given by Barger and DeLamater (1948). It employs thionyl chloride ($SOCl_2$) in place of the more usual sulphite compounds, and this breaks down to give a steady supply of SO_2.

$$SOCl_2 + HOH \rightarrow SO_2 + 2HCl$$

This method yields a remarkably stable solution whose only drawback appears to be that with certain tests, such as the performic acid or peracetic acid-Schiff tests for unsaturated lipids (Chapter XI, p. 325), colour is developed less readily than with less stable modifications. The de Tomasi-Coleman and the thionyl chloride methods for preparing Schiff's solution are given in Appendix 8, p. 822, together with the procedure of Itikawa and Ogura. The last of these is recommended for general routine use.

It has sometimes been suggested that basic fuchsin should be used instead of Schiff's reagent for demonstrating the aldehydes produced by the Feulgen hydrolysis (DeLamater, 1948 ; Arzac, 1950). The disadvantage of this modification lies in the fact that, unless a long staining period is employed, the aldehyde-*basic fuchsin* linkages (Schiff bases) cannot withstand alcoholic dehydration. This is in sharp contrast to the extreme stability of the aldehyde-*fuchsin-sulphurous acid* compound in all the usual solvents. Basic fuchsin is not recommended as a substitute for Schiff's reagent in any of the tests in which the latter is customarily employed.

Alternative Basic Dyes in Preparation of Schiff's Reagent. A dilute aqueous solution of thionin, decolourized with SO_2, was used by Östergren (1948) in place of Schiff's reagent for hydrolysed plant nuclei and DeLamater *et al.* (1955) noted that thiazine and oxazine dyes would produce substitutes for Schiff's reagent. Van Duijn (1956) used a modification of DeLamater's thionyl chloride-thionine in which the dye concentration was reduced to 0·05 per cent. This stained hydrolysed nuclei blue, but unhydrolysed nuclei also stained to some extent, probably because acid hydrolysis was produced by the staining bath itself. These alternative methods have largely been used for double staining techniques and they have little application at present in routine histochemistry.

Substitutes for HCl in the Feulgen Reaction. Alternative reagents for this purpose are fairly numerous. Widström (1928) used citric acid and De Lamater *et al.* (1950) used N-H_2SO_4 or HNO_3. Di Stefano (1952) preferred perchloric acid and Hashim (1953) phosphoric acid. Later additions to the list include 5 N-HCl and 5 N-HNO_3 (Itikawa

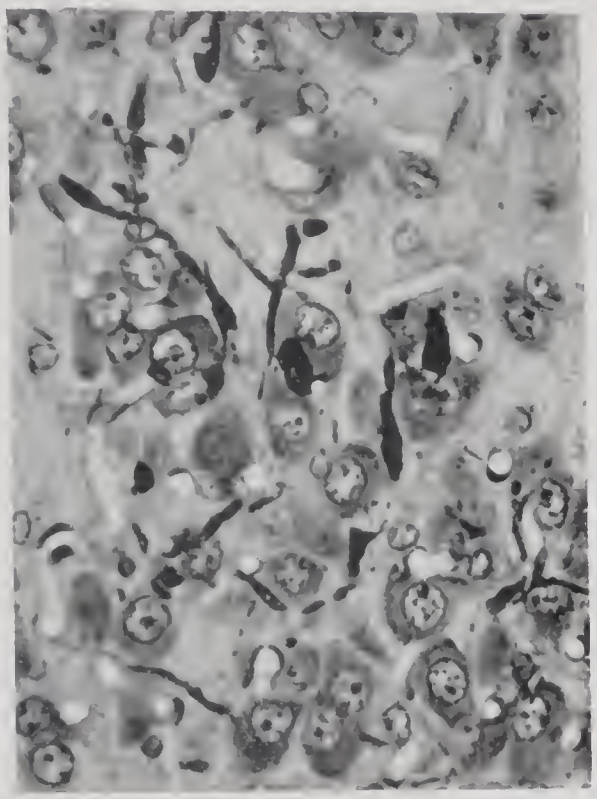


FIG. 61. Rat hypothalamus. Paraventricular nucleus. Shows "axons" charged with "neurosecretory substance". Chrome alum-haematoxylin-phloxin. × 370.

FIG. 62. As Fig. 61. Following incubation with ribonuclease. Chrome alum-haematoxylin-phloxin. × 1350.

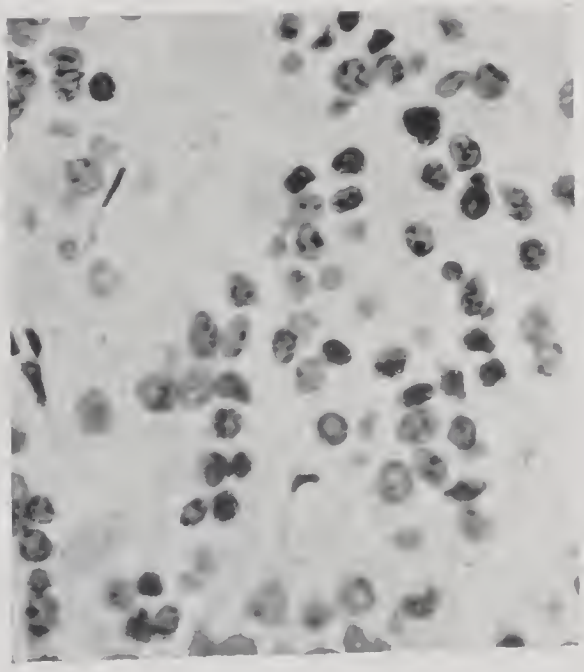


FIG. 64. Nuclei stained by the Feulgen method for thymonucleic acid (Schiff's reagent after N HCl at 60° for 8 minutes). Only chromatin stains purple. × 520.

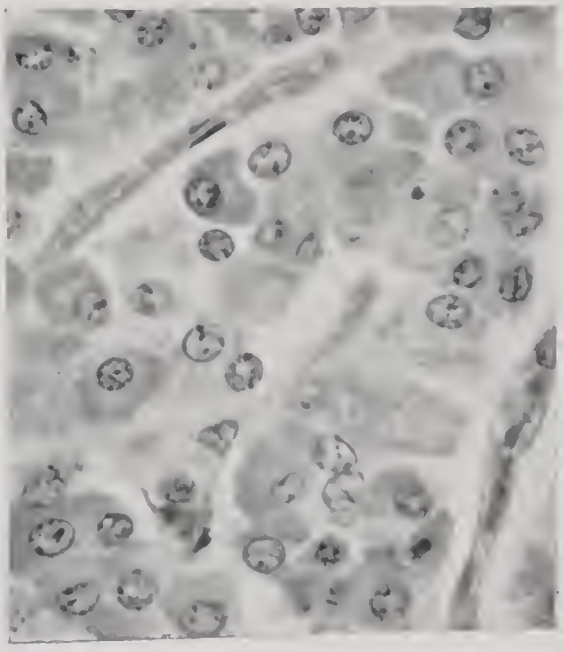


FIG. 65. Serial section to Fig. 64. Nuclei stained by 2-hydroxy-3-naphthoic acid and diazotized *o*-dianisidine after N HCl at 60° for 8 minutes. Chromatin purple, cytoplasm pink. × 520.

[*To face p.* 203.

and Ogura, 1954), trichloroacetic acid (Bloch and Godman, 1955) and bromine in carbon tetrachloride (Barka, 1956). The last alternative is the most interesting although the author considers that the mechanism is the same as that of the standard Feulgen reaction. No real advantages over the latter have been demonstrated in any of the above-mentioned variants.

The Plasmal Reaction. Interference with the performance of the Feulgen reaction occurs through the so-called plasmal reaction of Feulgen and Voit (1924). This is due to the presence in the tissues of labile acetal phospholipids, whose aldehyde groups are easily revealed by mild hydrolysis or oxidation, after which they react with Schiff's reagent to give the usual red dye. In paraffin sections this interference is minimal but it is much greater in frozen sections. Its importance in frozen sections is presumably the reason why the subject appears frequently in the continental literature, but hardly at all in English and American work. Acetal phospholipids (plasmalogens) may be removed from sections by prolonged immersion in 90 per cent. alcohol or by treating the sections with a solution of hydroxylamine hydrochloride in sodium acetate (Appendix 12, p. 865) which converts them to oximes. These acetal phospholipids are considered more fully in Chapter XII.

The Feulgen-naphthoic Acid Hydrazide Reaction

Complications due to the use of Schiff's reagent for revealing aldehydes produced in the nuclei by mild acid hydrolysis can be avoided by the use of a different reagent for this purpose. Danielli (1947) used 2 : 4-dinitrophenylhydrazine, which stained the chromosomes yellow. He concluded that this result confirmed those given by Schiff's reagent. Better results are obtained by using 2-hydroxy-3 naphthoic acid hydrazide (Pearse, 1951). This reagent, first synthesized by Franzen and Eichler (1908), was used by Camber (1949) for the localization of ketosteroids in the adrenal cortex. It combines with aldehydes as well as ketones and the resulting compound, which is dark yellow in colour, is coupled with diazotized *o*-dianisidine in alkaline solution to give a purplish-blue compound. By this method the localization of chromatin staining in the nuclei is precisely similar to that shown by the true Feulgen reaction (Figs. 64 and 65). Cytoplasmic detail is visible in Fig. 65 because the hydrazide reagent combines with tissue proteins by virtue of other groups in its molecule and the subsequent treatment imparts a pinkish colour to these protein hydrazides. Although this does not provide a complete confirmation of the specificity of the Feulgen-Schiff reaction for the location of DNA, if the appearances in the latter were due to diffusion of the soluble dye product (aldehyde-fuchsin-sulphurous acid,

different appearances might be expected to occur with the use of so different a compound as the naphthoic acid hydrazide. The solubility of the aldehyde-hydrazide compound doubtless differs from that of the aldehyde-fuchsin-sulphurous acid compound and, while the latter is a basic dye with affinity for acid substances (such as nucleic acids), the former combines with tissue proteins by virtue of the hydroxy-naphthoic acid part of the molecule and here probably acts as an acid "dye."

Miscellaneous Methods

There are several biochemical methods for DNA, depending on the presence of 2-deoxyribose, which have a high degree of specificity. These are the carbazole and diphenylamine reactions of Dische (1930, 1944) and the tryptophan-perchloric acid condensation method of Cohen (1944), which was employed for the demonstration of DNA in normal and pathological sera by Seibert *et al.* (1948). The carbazole reaction, which Schneider (1948) considers specific for the pyrimidine-bound aldehyde of DNA, and the diphenylamine reaction are both carried out in acid solutions with heating to 100°, the former in 85 per cent. sulphuric acid and the latter in glacial acetic acid. They have not been successfully modified for histochemical use. The method of Cohen depends on the condensation of a secondary amine (tryptophan) with deoxyribose freed from purine and pyrimidine groups by treatment with hot perchloric acid. This condensation takes place slowly even in the test tube and cannot be produced *in situ* in tissue sections since the removal of DNA by hot perchloric acid takes place too rapidly.

Staining of DNA and RNA with Basic Dyes

General Considerations

The primary PO_4 groups of the nucleic acids have a pK in the region of 2, according to Jordan (1952), and above pH2 they therefore carry a negative charge and combine with cationic groups of the basic dyes. It is usually considered that union of the latter with nucleic acids is by salt linkages but recent evidence, considered below, suggests that hydrogen bonding may be involved. In the tissues the nucleic acids are present as nucleoproteins and a proportion of their PO_4 groups is bound to protein (protamine, histone). Displacement of the latter frees phosphate groups for combination with basic dyes. The ratios of arginine and lysine to PO_4 are constant (Butler *et al.*, 1955) but the total amount of basic amino-acid is usually 10 per cent less than the total amount of phosphate so that some of the latter remains unbound to histone. Knoblock and Vendrely (1956) found a constant ratio

(5·0) between DNA and arginine in fish erythrocyte nuclei but in the sperm of some species (*Trutta*, *Esox*) it was much lower (1·6). In *Cyprinus* and *Tinca* the ratio in sperm was the same as that of the somatic nuclei.

A number of investigators have studied the problem of dye binding by nucleic acids in solution and in tissue sections. Hermann *et al.* (1950), using solutions and sections of muscle, found that between pH4 and pH6 the binding of *toluidine blue* was a function of the RNA content. Kurnick and Mirsky (1950) reported stoichiometric precipitation of polymerized DNA by *methyl green* and Kurnick (1950a) obtained a similar result *in vitro* with crystal violet. Irvin and Irvin (1949, 1952) studied the binding of *aminoacridines* and found that under certain conditions depolymerization resulted in decreased binding of the dye. Similar reports have been made for 5-aminoacridine (Lawley, 1956a) *quinolines* (Parker, 1949), methyl green, *ethyl green* and *malachite green* (Kurnick, 1950a and c). On the other hand Lawley (1956b) and Cavalieri and Angelos (1950) reported a decreased uptake of *rosanilin* by depolymerized DNA. Lawley also found that metal cations (Mg, Ba) could compete with dyes for binding sites but that their effect was greater (30×) for rosanilin than for 5-aminoacridine. This observation agrees with those of De Bruyn *et al.* (1953) who suggested that the acridines (particularly 2, 8-diaminoacridine) possess NH_2 groups correctly spaced for hydrogen bonding with the PO_4 groups of DNA. These are approximately 7-8 Å apart whereas the NH_2 groups of rosanilin are 10 Å apart.

It is evident that relatively minor changes can alter very greatly the uptake of basic dyes by nucleic acids.

The Ribonuclease, Methyl Green-Pyronin Method (Brachet)

The standard method for demonstrating RNA in both nucleus and cytoplasm is the indirect method evolved by Brachet (1940a, 1942, 1944), which depends on the specific depolymerization of RNA by the enzyme ribonuclease. This treatment does not affect DNA in nuclear chromatin or elsewhere. Brachet showed that the methyl green of Pappenheim's (1899) mixture was an elective stain for DNA in chromatin, while the pyronin G of this mixture had an elective affinity for RNA in both nucleolus and cytoplasm. With Brachet's method of two parallel sections, therefore, one is exposed to ribonuclease and then both are stained with methyl green-pyronin. Alternatively, staining may be carried out with 1 per cent. aqueous toluidine blue, with or without counterstain, or with 1 per cent. toluidine blue in 95 per cent. ethanol which is recommended by Kurnick (1952) as a rough screening test for nucleic acids. Material staining blue with toluidine blue or red with pyronin, and removable by treatment with ribonuclease,

is considered to be RNA; that which is not so removable is not. The Pappenheim method is probably the method of choice for investigating the RNA content of the majority of tissues, although it does not always allow critical appreciation of cytological detail. If this is required, separate sections must be stained by some wholly cytological method or with toluidine blue followed by a few seconds staining with 2 per cent. orange G in 5 per cent. phosphotungstic acid. This last method shows both RNA and cytological detail. If other types of nucleic acid extraction are used (see below) staining with toluidine blue is preferable to the use of the Pappenheim method.

Some Important Results of the Methyl Green-Pyronin Method. The use of this method, especially in the hands of its originator, has greatly increased our knowledge of the relationship between the two nucleic acids in the nucleus and RNA in the cytoplasm. Most of the important papers of Brachet on this subject are given in his book (1950), a second edition of which has now appeared (1958). Two papers which may be mentioned specifically (1940a, 1940b) refer to the use of the method for determining RNA in various tissues of the frog. Desclin (1940) studied the hypophyses of rats and guinea-pigs and found RNA in the basophils and Dustin (1942) found RNA in mammalian and reptilian reticulocytes. Biesele (1944) added some important findings in the case of skin tumours produced in mice by the action of methyl cholanthrene. Roskin and Ginsberg (1944) applied the method to protozoa, particularly to plasmodia and trypanosomes, and Dempsey and Wislocki (1945) examined human placental syncytium. Mottram and Selbie (1945) examined the distribution of RNA in bean roots and in Rous sarcomas in chickens, *inter alia*. Dempsey and Singer (1946) found ribonuclease-resistant material in the Z line of mammalian muscle. Stowell (1946b) and Deane (1946) investigated RNA in various human tumour cells and in normal human liver cells, respectively, and numerous authors have applied the method to bone marrow smears (Brachet, 1942; van den Berghe, 1942; White, 1947). Smears of the peripheral blood have also been stained by the methyl green-pyronin method (Perry and Reynolds, 1956) and it has been used for further studies in cancer cytology (Hopman, 1956). Laverack (1955) recommended the technique for application to frozen sections and it is certainly one of the better methods for studying nucleic acids in unfixed (cold microtome) sections. Barka *et al.* (1953) used the method on tissue cultures of chick embryo liver prepared by the hanging drop method. Their investigations showed a close correlation between the capacity of the cells to produce RNA and their capacity to proliferate in tissue culture.

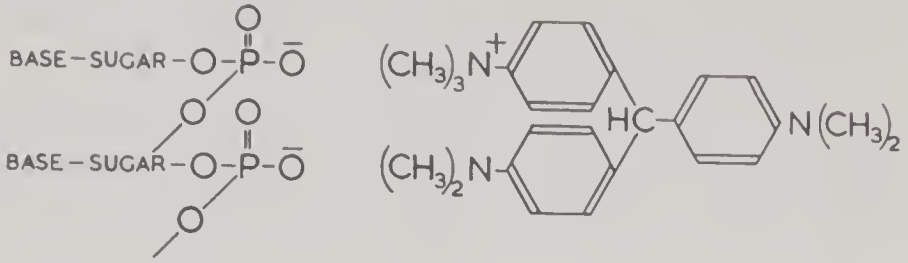
The papers quoted above represent only a fraction of those published in which the Brachet method has been used. They are mainly

those in which the method was the only important one employed. The volume of papers on the subject alone indicates its importance and the method remains to-day as standard histochemical practice for all investigations involving the distribution of the nucleic acids.

Theoretical and Practical Considerations of the Methyl Green-Pyronin Technique. Kaufmann *et al.* (1948), using sections or smears fixed in acetic alcohol and stained with methyl green-pyronin, found that the chromosomes were blue-lavender in colour, while the nucleoli, and the cytoplasm of cells containing RNA, were red. After ribonuclease no component colourable with pyronin remained, so that the cytoplasm and nucleoli were colourless while the nuclei were green. This suggested that RNA was present in the chromosomes. If sections were treated with pepsin, and subsequently stained with methyl green-pyronin, chromosomes, nucleoli and cytoplasm all stained more intensely with pyronin than in control sections. After successive treatment with pepsin and ribonuclease, chromosomes, cytoplasm and nucleoli no longer stained red, indicating that ribonuclease had removed the pyronin-stainable material. Kaufmann *et al.* suggested that RNA was present in cells, either free or combined as ribonucleoprotein, and that its affinity for dyes was influenced by the state of association. It has also been considered (Kurnick, 1947, 1949, 1950a and b) that the difference in stainability of chromosomes and cytoplasmic nucleoprotein may be a matter of the degree of polymerization. This author, using the same two dyes together and separately, to stain DNA and RNA *in vitro*, found that highly polymerized DNA stained with pyronin about one-fifth as intensely as RNA and that DNA-histone stained about one-sixth as intensely. DNA which had been depolymerized, however, stained as strongly as RNA with pyronin. Using the two dyes together, RNA stained pale pink, depolymerized DNA a similar colour, and polymerized DNA green. Kurnick concluded, therefore, that pyronin distinguished only different states of polymerization of the nucleic acids rather than chemical differences between the two acids.

Vercauteren (1950), while agreeing with Kurnick's conclusions, considered that less drastic changes in structure than depolymerization could cause DNA to lose its affinity for methyl green. Using unfixed smears of thymus nucleohistone he found that 5 minutes' treatment with N-HCl at 60° would destroy its affinity for methyl green while enhancing that for pyronin. Short immersion in veronal-acetate buffer at pH 1·6, or in neutral solutions of formalin, had a similar effect. Vercauteren claimed that the stereochemical factor in the nucleic acid molecule which accounted for its high affinity for methyl green was the presence of negatively charged phosphate residues at a

distance corresponding to that between two possible sites for positive charges on the methyl green molecule.



He suggested that the effect of dilute acids at low temperatures was sufficient to break certain weak (hydrogen) bonds in the DNA molecule, causing a coiling of the molecule to occur. This coiling would alter the distance between adjacent PO_4 residues which would thus no longer fit the methyl green molecule.

Opposition to Kurnick's hypothesis has come mainly from Taft (1951b) and Alfert (1952). The former treated tissues at pH3 and pH11·7 and at 1° and 24° to produce depolymerization of DNA, without affecting methyl green staining. It is probable, however, that depolymerization of this type, which is due merely to rupture of hydrogen bonds (Gulland and Jordan, 1947), is reversible. Nuclear staining with methyl green was considered by Alfert to be at least partly due to blocking of the stainable groups in RNA by proteins and he considered also that the staining of DNA by this dye was subject to variations in degree due to varying amounts of protein binding. There is, however, no essential disagreement between these findings and Kurnick's observations, and most workers are prepared to agree that under carefully controlled conditions methyl green staining is selective for polymerized DNA. As pointed out by Godman and Deitch (1957), however, even when methyl green is used with the precautions advised by Kurnick (1955b) it cannot by itself afford unequivocal evidence of depolymerization of DNA. Godman and Deitch showed that acetylation, which blocks the positively charged ϵ-amino groups of lysine and the guanidyl groups of arginine, restored the methyl green staining of LE bodies in systemic lupus to figures equivalent to those derived from Feulgen staining.

Many methods for standardizing the performance of the methyl green-pyronin stain have appeared in the past ten years. One, described by Taft (1951a and b), depends on repeated chloroform extractions of a 0·5 per cent. solution of methyl green in 0·1 M-acetate buffer, to which is subsequently added 0·2 per cent. pyronin B. Dehydration is carried out in a mixture of 3 parts of tertiary butyl alcohol and 1 part ethanol, and clearing and mounting as usual. A second method for preparing the methyl green-pyronin stain (Trevan and Sharrock, 1951)

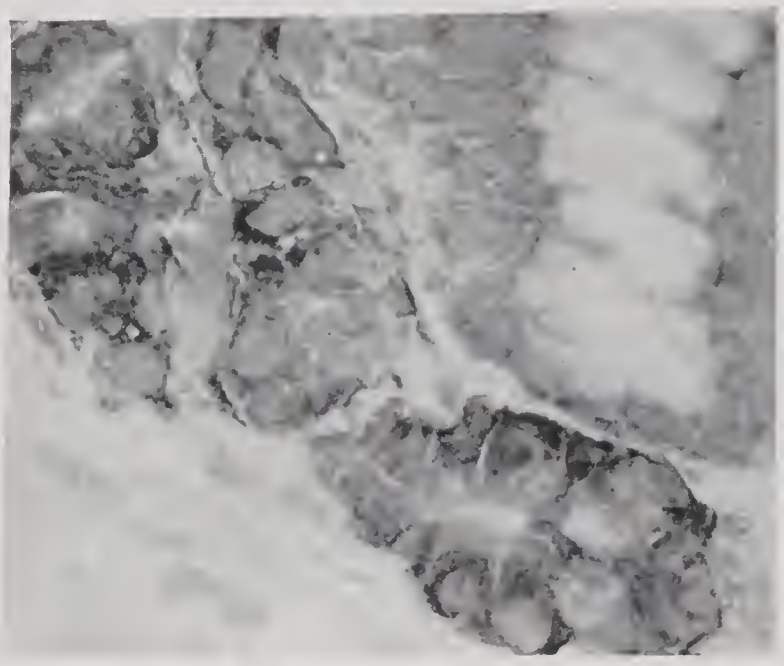


FIG. 66. Newt (*Triton*). Digestive tract. Fresh frozen section fixed in aceticethanol. Nuclei green, cytoplasmic ribonucleoprotein red. Methyl green—pyronin. × 400.

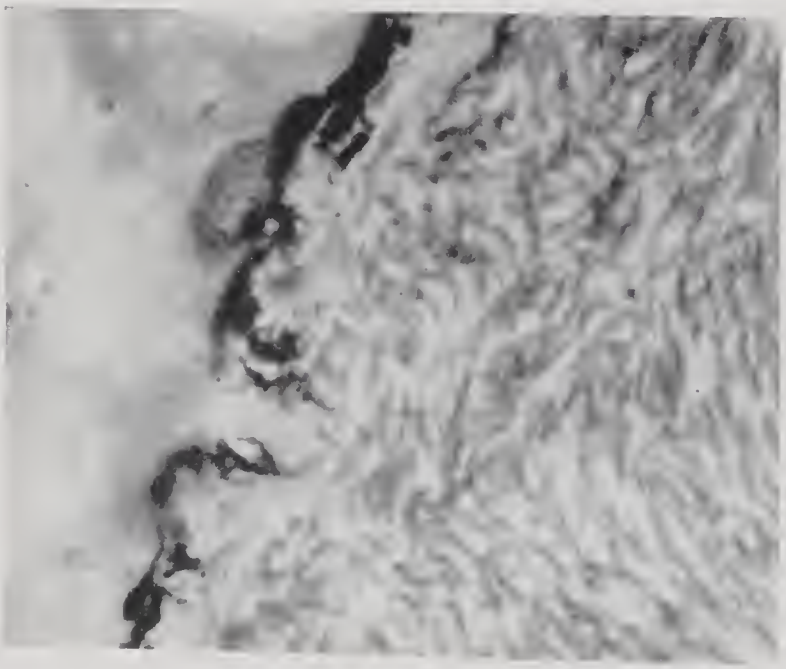


FIG. 67. Snail (*Helix aspersa*). Testis. Shows (right) green stain heads of spermatozoa and (left) pigment layer with supporting cells containing much red-stained RNA. Methyl green—pyronin. × 930.

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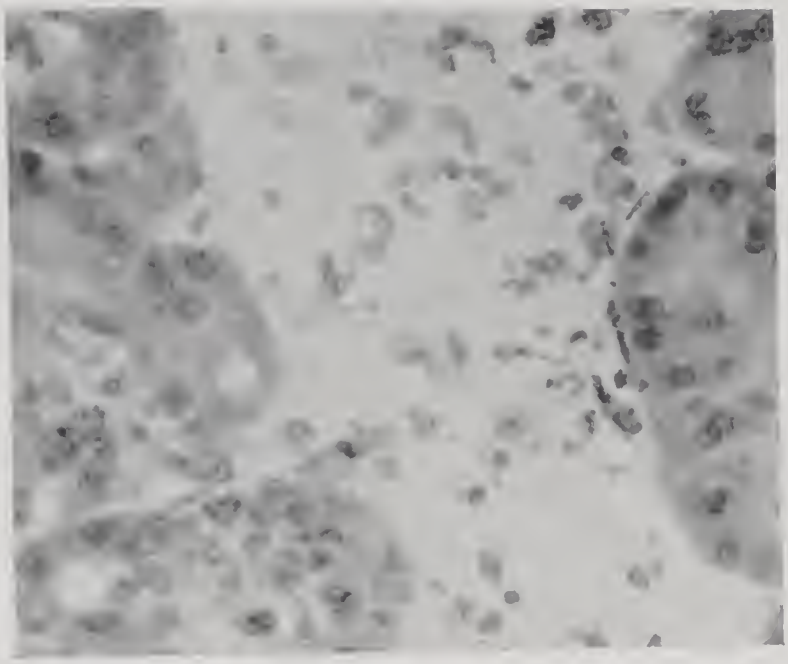


FIG. 68. Rat pancreas. Acinar cells contain much RNA (red). Connective tissue and acinar cell nuclei bright green. Methyl green—pyronin. × 400.

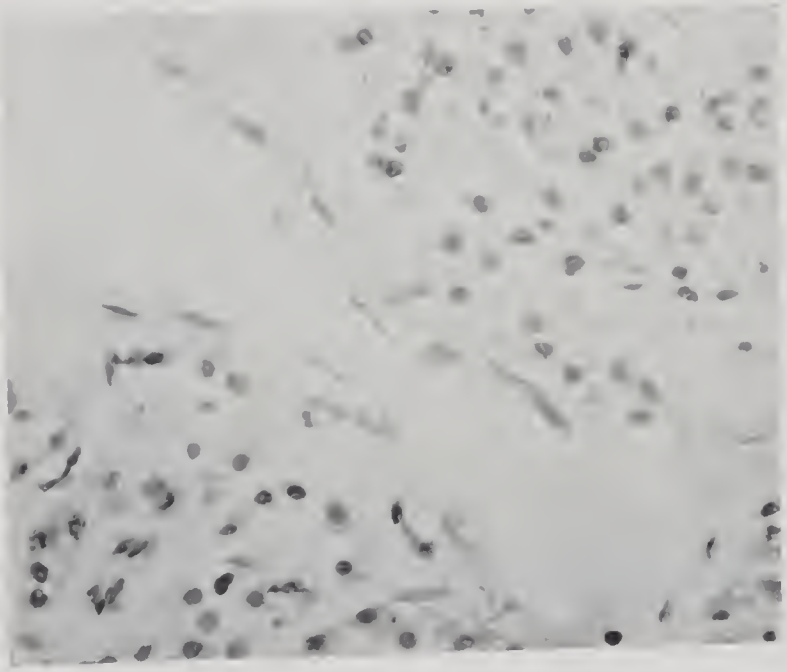


FIG. 69. As Fig. 68. After 1 hour's treatment with ribonuclease in distilled water. Only the nuclei are stained (green). Methyl green—pyronin. × 400.

is given in Appendix 8, p. 825 and a third (Kurnick, 1955b) is given immediately after this. This method, in which chloroform-washed pyronin-Y is used, is more selective for RNA than other existing methods. A valuable discussion of the theory and practice of methyl green (and methyl green-pyronin) staining appears in Kurnick's (1955a) review of the histochemistry of the nucleic acids, and the type of result available is illustrated in Figs. 66, 67, 68 and 69. Technical and theoretical points concerning the use of ribonuclease are discussed in Chapter XXII.

Quantitative Histochemical Estimation of Nucleic Acids

Three methods have been described for the quantitative estimation of nucleic acids, two applicable solely to DNA and the third to total nucleic acid irrespective of type.

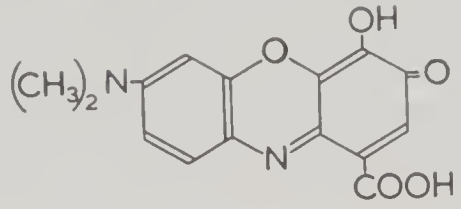
The first method, which has already been mentioned briefly, is that of Stowell (1942) who described a relatively simple apparatus for the photometric measurement of the amount of colour developed in nuclei stained by the Feulgen reaction. Measurements were made of the transmission of light through stained and unstained sections and through portions of the glass slides away from the sections in both cases. The method depends on a proportional correlation between the amount of the stain present and the substance for which it is specific, a relationship which has not been elucidated in the case of the Feulgen reaction.

More recently Kurnick (1949) has described a method depending on the application of spectrophotometry to nuclei stained with methyl green. Tissues for examination are immersed in 30 per cent. sucrose, as described by Hogeboom *et al.* (1948), before fixation in neutral formalin. This process yields nuclei which are homogeneous except for the nucleoli. After removal of histones by treatment with N-HCl, sections are stained for 14 hours at 4° in a 0·25 per cent. solution of methyl green in 0·2 M-acetate buffer at pH 4·1, the solution being chloroform-washed before use (see Appendix 8). After staining, the sections are mounted in buffer and the coverslips ringed with paraffin wax. Photometry is carried out at 645 mμ. Using this method the author found $0{\cdot}6 - 0{\cdot}8 \times 10^{-8}$ mg. DNA per nucleus in calf thymocytes, values in close agreement with those found in chemical analysis and by densitometry of photographic plates of thymus nuclei photographed at several different wavelengths in the ultraviolet.

Kurnick criticized the method of Pollister and Leuchtenberger (1949), who made a photometric assay of sections of mouse liver stained with methyl green, on account of the high staining temperature employed (56°) and because of their use of alcohol for differentiation. These criticisms are valid but Kurnick's method itself is also open to

criticism. Much depends on the exact ratio in which methyl green combines with the nucleic acid. In calculations made from his original figure (dye-P ratio 1 : 10) certain differences were present between the theoretical and the observed coefficients of extinction. Kurnick suggested that these discrepancies might be due to the combination of DNA with proteins other than histones, which would consequently fail to be removed by treatment with N-HCl, but found that in practice competing protein was not a problem. Further investigation of the stoichiometry of methyl green staining revealed that the dye-P ratio of 1 : 10 was given by heptamethylpararosanilin (C.I. 684) and that the ratio 1 : 13, quoted in an addendum to the paper, was produced by the closely related dye hexamethylethylpararosanilin (C.I. 685).

More recently still Einarson (1951), in the latest of a long series of publications on the use of the chromalum lake of gallocyanin as a basic dye (Einarson, 1935, 1936, 1949), has proposed a quantitative method using this dye. Gallocyanin is an oxazine dye which acts in aqueous solution as a weakly acid stain.



When mixed with chromalum, however, it forms three salts which Einarson refers to as lake-cation [gallocyanin-Cr $(H_2O)_4$], lake-hydroxide [gallocyanin-Cr $(H_2O)_4OH$], and lake-sulphate [gallocyanin-Cr $(H_2O)_4]_2SO_4$. The compound responsible for staining the nucleic acids is the red-coloured lake-cation which combines with acid groups, particularly with the phosphate groups of the nucleic acids, to form a dark blue lake-tissue salt. Einarson (1951) stated that the reaction between gallocyanin-chromalum-lake-cation and the nucleic acids was sufficiently specific for use with photometric or densitometric estimations of the degree of basophilia. " From the light transmitted the relative values of the quantity of nucleic acid may be deduced." Although the author discussed at great length the mechanism of his gallocyanin staining, and although he undertook measurements of the type described, until more information is available about the proportions in which gallocyanin combines with the phosphate groups of the nucleic acids (of the type which Kurnick gave in respect of the similar use of methyl green) the method will not be applicable to histochemistry on a strictly quantitative basis. These remarks must still stand, although Sandritter *et al.* (1954) have now reported that *in vitro* 1 molecule of gallocyanin is bound by 15 phosphorus atoms in " polymerized " RNA and by 23 atoms in heated RNA. The stoichio-

metry of DNA was not reported by these authors, who did not give any information about the application of their findings. Diefenbach and Sandritter (1954), however, stated that GC combined stoichiometrically with DNA, the ratio GC to nucleic acid being 1 : 3·7.

Gallocyanin-chromalum for DNA and RNA

For the simple staining of nucleic acids gallocyanin-chromalum (GC) possesses two features of considerable value. It is a progressive stain, and it withstands alcoholic dehydration and clearing in xylene. Staining can be carried out at any pH between 0·8 and 4·3, but it is particularly at the lower levels, between pH 1·5 and 1·75, that specific staining of nucleic acids is at its highest and non-specific staining is negligible. The latter is considered to be due to attachment of the lake-sulphate to acid groups in the tissues through its dimethylamine grouping, $(CH_3)_2N$—. The specificity of the GC method for nucleic acids was investigated by Stenram (1953, 1954) who found that he could still obtain staining of Nissl substance in nerve cells after ribonuclease extraction although sections stained with toluidine blue or methylene blue showed no staining at all. This result occurred using GC at pH 1·0 or at any pH up to pH 4·0. Stenram suggested that the non-specific staining might be due to a separate (possibly removable) component, and subsequent work by de Boer and Sarnaker (1956) has confirmed this. These authors extracted from aqueous solutions of gallocyanin a component which gave bright blue staining of Nissl bodies with no staining of other cytoplasmic components. Mayersbach (1956) considered the GC method to be more reliable than methyl green-pyronin and he preferred it on that account, for the demonstration of RNA. I do not consider that it can in any way take the place of the methyl green-pyronin method but recommend it as a method of high specificity, involving no differentiation, which is relatively insensitive to differences in fixation. Details of the GC method as a progressive stain and instructions for its preparation are given in Appendix 8, p. 826.

Extraction Techniques for Nucleic Acids

During the past few years a number of extraction techniques for nucleic acids, derived from chemical procedures in the first instance, have been applied to histochemistry. Most of the methods extract both acids, but at different rates, and they vary in the degree of specific removal of one or both nucleic acids which they are able to achieve.

Sodium Chloride Solutions

It has long been known that certain concentrations of sodium chloride remove the basophilic material from the cytoplasm while

leaving the nuclei intact. Levene (1901) used sodium or ammonium chloride solutions to extract nucleic acids from cells and his lead has been followed, after a long interval, by numerous workers in this field. Mirsky and Pollister (1942, 1943, 1946), for instance, found that ribonucleoprotein and deoxyribonucleoprotein could be extracted from liver by 0·15 M (0·85 per cent.) and 1·0 M-NaCl, respectively. The removal of these substances was thought to be due to the depolymerizing effect of salt solutions on nucleohistones. Opie and Lavin (1946) found that 1·0 M and 0·5 M-solutions of sodium chloride did not affect cytoplasmic basophilia but that some diminution occurred in 0·33 M and 0·2 M concentrations; 0·17 M-NaCl (0·95 per cent.) achieved almost complete removal of RNA from sections fixed in acetic-alcohol-formalin, or in formalin alone, the sections being immersed for 5 hours at 37° or for 2 hours at 56°. White (1950), using human liver smears wet-fixed in Susa, demonstrated these actions cytologically by staining the smears, previously extracted with saline, in aniline blue-orange G mixtures at pH 2–3. He found that with the lower concentration of NaCl the blue-staining cytoplasmic ribonucleoproteins were replaced by protein with an affinity for orange G. After 1·0 M-NaCl solutions a similar effect was noticeable in the nuclei.

Perchloric Acid

After initial work by Ogur and Rosen (1949), who used perchloric acid for the extraction of both nucleic acids from onion root tip material, Erickson *et al.* (1949) evolved a method for the extraction of RNA from alcohol-fixed sections. They employed 10 per cent. aqueous perchloric acid at 4° for periods of between 4 and 18 hours, and found that the longest period invariably removed all cytoplasmic basophilia, using 1 per cent. aqueous toluidine blue as an indicator. These authors also used 10 per cent. hot perchloric acid (70°) for up to 20 minutes to extract both DNA and RNA from sections. The method was employed by Sulkin and Kuntz (1950) on Zenker-fixed mammalian tissue sections, followed by Mallory's phloxin-methylene blue stain. Erickson and his co-workers, and Sulkin and Kuntz, both considered that perchloric acid could be substituted for ribonuclease in the histochemical detection of ribonucleic acid. The former authors stated that they obtained " comparable," and the latter " identical," results using ribonuclease on the one hand and cold perchloric acid on the other. Seshachar and Flick (1949), however, employed perchloric acid in varying strengths to extract RNA from protozoan cells fixed in 1 : 3 acetic acid-alcohol, and concluded that only under rather rigid conditions could perchloric acid imitate ribonuclease. They found that 2 per cent. perchloric acid, in the cold, had no effect on the intensity of the Feulgen reaction, but that it appeared to depolymerize

DNA progressively as judged by the progressive decrease in staining with the methyl green component of Pappenheim's stain. Parallel with the decrease in methyl green staining a rise in affinity for pyronin occurred in the structures concerned. As already noted, Kurnick (1947) believes that this affinity for pyronin indicates the presence of nucleic acid in a lower degree of polymerization, basing his assumption on the fact that pyronin stains only RNA and depolymerized DNA *in vitro*. Seshachar and Flick found that 10 per cent. perchloric acid, in the cold, removed RNA very rapidly; moreover, it progressively diminished the intensity of the Feulgen reaction and the affinity of chromatin for methyl green. The accumulated results, obtained by these authors, appear in Table 13, below.

TABLE 13

Per cent. $HClO_4$	Temp. °C	Time (hours)	Result	
			Cytoplasm	Nucleus
2	5	3, 10, 18, 24, 72	Progressive diminution of basophilia from 10 hours	Feulgen unchanged. Methyl green diminished from 18 hours
10	5	3, 10, 18, 24, 72	No basophilia	Feulgen diminished. Methyl green nil at 72 hours
5–10	35	18	No basophilia	Feulgen and methyl green very faint
5–10	70	20	No basophilia	Feuglen and methyl green nil

My experience, on human and animal tissues fixed in various fixatives, agrees with that of Seshachar and Flick. I do not consider that parallel results can be obtained with ribonuclease and perchloric acid. Ribonuclease, freed from proteolytic activity, removes only RNA from sections. Perchloric acid extraction certainly removes all RNA, but it also extracts various protein, glycoprotein and lipoprotein materials and progressively depolymerizes DNA. Fig. 70 shows a group of plasma cells with large amounts of pyronin-positive RNA in the cytoplasm; the nuclei stain purple (methyl green-pyronin). Fig. 71 shows a similar section extracted with cold perchloric acid. Cytoplasmic RNA has vanished but the nuclei now stain red (pyronin only), indicating depolymerization of DNA. This does not mean that perchloric acid is not a useful substitute for ribonuclease, as found by Wenderoth (1953) in the case of peripheral blood and bone-marrow smears, but that the choice of staining may subsequently be somewhat limited. It would appear, from Seshachar's and Flick's results, that there should be a period of some hours between the point of maximal RNA extraction and the beginning of depolymerization of DNA as

evidenced by the falling off in affinity for methyl green. Goessner (1954) investigated this point and found, as did Franz *et al.* (1954) and Koenig and Stahlecker (1951, 1952), that fixation had a considerable effect on the amount of stainable RNA after various times of extraction.

On the whole, it seems likely that the now universal availability of ribonuclease has made the use of substitutes like cold perchloric acid unnecessary.

Bile Salts

Henry and Stacey (1943), in their experiments on the nature of the Gram-positive complex in micro-organisms, employed solutions of bile salts to remove what was subsequently demonstrated to be magnesium ribonucleate from the Gram-positive bacterial cytoskeletons. They used 2 per cent. aqueous sodium cholate at 60° in the presence of oxygen. Two to three hours' incubation was sufficient to remove the RNA from the majority of cells. No explanation for this effect of bile salts appears to have been offered, but it has been stated that it is not due to lowering of surface tension. Foster and Wilson (1951, 1952) studied the effect of bile-salt extraction on the Gram-positive staining of the pituitary β granules, which they considered might be due to the presence of RNA. With this process they were able to destroy both Gram-positiveness and basophilia in the β granules, although they were able to reverse only their basophilia by means of hydrolysis with ribonuclease. It is probable that the bile salt extraction technique will become a more useful one in histochemistry when something more is known of the types of materials removed and of the optimum duration of incubation. Little further work on this subject has been reported, however, with the exception of a chemical study by Kay and Dounce (1953).

Trichloroacetic Acid

Cohen (1944) used trichloroacetic acid for the extraction of DNA and Schneider (1945), with whose name the process is usually associated, employed a 5 per cent. aqueous solution at 90° for 15 minutes to extract nucleic acids from the tissues. Both deoxyribonucleic and ribonucleic acids are removed by this procedure, which has been applied by Kaufmann *et al.* (1950) to onion root tips and by White (1950) to bone marrow smears. Both these authors have shown that Schneider extraction leaves behind a protein component of the nucleus which can be demonstrated by its increased affinity for acid dyes. Kaufmann *et al.* used fast green and White a mixture of aniline blue and orange G at pH2–3. The latter showed that the globin of the erythrocytes, and to a lesser extent the intranuclear histones, retained orange G, while the plasma proteins and cytoplasmic proteins, including those

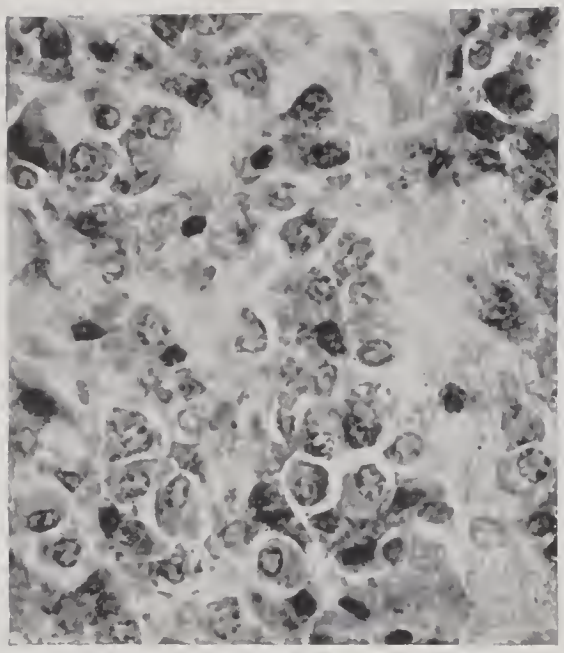


FIG. 70. A group of plasma cells (human with purplish-green nuclei and red cytoplasmic granules. Pyronin-methyl green. × 520.

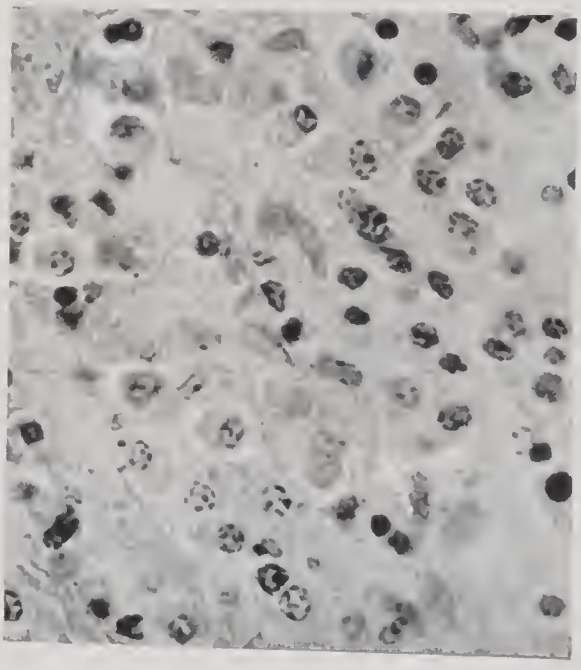


FIG. 71. The same group of plasma cells shown in Fig. 70, in a section treated with 10 per cent. perchloric acid at 4° for 16 hours. Ribonucleic acid has completely disappeared. The nuclei, green after ribonuclease, are red in this instance. Pyronin-methyl green. × 520.

[*To face p.* 214.

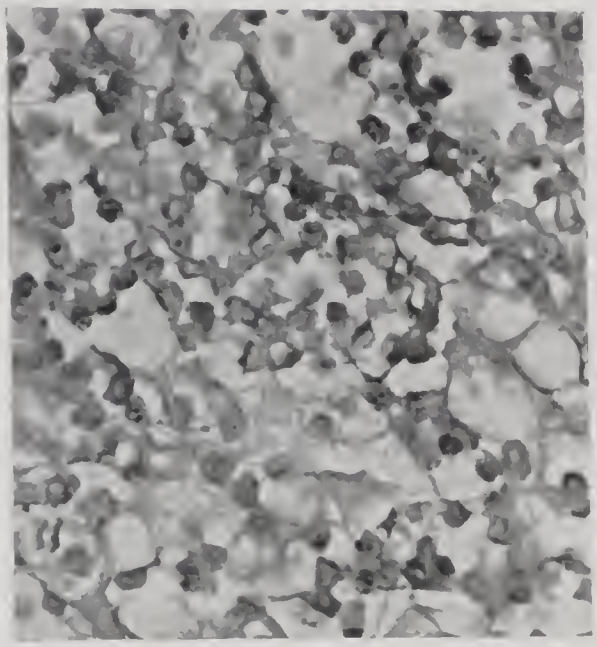


FIG. 72. A group of plasma cells (human), showing the small affinity of their nuclei for acid dyes. Light green S.F. × 520.

FIG. 73. Serial section to Fig. 72, treated with 5 per cent. trichloroacetic acid at 90° for 15 minutes (Schneider extraction). Stained and photographed as Fig. 72. Light green S.F. × 520.

coupled to RNA, stained more slowly with aniline blue. Figs. 72 and 73 illustrate the effect of Schneider extraction on plasma cell nuclei, Helly-fixed, stained with 1 per cent. aqueous light green SF.

Other Methods

Constantin Saint-Hilaire (1898), in his researches on nucleohistones, used water to extract these substances from unfixed tissues. Mann (1902) showed that water would not remove nucleic acids from fixed tissues but that if acetic acid-fixed material was treated for a short while with dilute hydrochloric acid nucleohistones were decomposed, leaving the histone *in situ*. More prolonged extraction removed the histones also. Hydrolysis in N-HCl at 60° for 10 minutes can usefully be employed to remove purines and pyrimidines from smears fixed in acetic-alcohol or from sections fixed in alcoholic fixatives. Slightly longer incubation may be necessary in the case of formalin and longer still if sublimate or chromates have been used. More recently, Sevag *et al.* (1940) and Davidson and Waymouth (1944) have used dilute HCl for nucleoprotein extraction, and normal HCl at 37° for 3 hours has been employed by Dempsey *et al.* (1950) as a means of reversing the tissue basophilia due to nucleoproteins of both types. This last procedure leaves the tissues in good condition for subsequent staining, unlike the more violent trichloroacetic acid method, but they do not stand up so well to further procedures involving heat or to analysis by treatment with enzymes. Caspersson *et al.* (1935) used malonic acid for the extraction of nucleic acids and formic acid has also been employed for this purpose. Neither of these techniques has any advantage for use in histochemistry.

Buffer Solutions

Brachet (1940a) noted that incubation in water at 70° progressively diminished the cytoplasmic basophilia due to RNA and subsequently numerous observers (e.g. White, 1947; Stowell and Zorzoli, 1947) described the extraction of RNA by various buffers, especially on the alkaline side of neutrality. To avoid these effects it is now customary to use depolymerizing and other enzymes in glass distilled water in order to escape the action of electrolytes, and at 37° to avoid the extractive effect of water at higher temperatures.

THE HISTOCHEMISTRY OF THE GRAM STAIN

Introduction

In 1884 Christian Gram described his aniline-gentian violet and iodine method of staining which has since been subjected to a large number of variations (the earlier ones listed by Hucker and Conn, 1923).

It is in bacteriology, of course, that the method finds its most important application in the division of micro-organisms into the two classes of Gram-positive and Gram-negative. As a histochemical reaction, difficulties in standardizing the technique and in interpretation of the results detract largely from its usefulness. Nevertheless, since great advances have recently been made in determining the mechanism of the Gram stain with reference to bacterial cells, it is pertinent to enquire how much these mechanisms apply to the staining of tissue components by the same method.

In histological practice the term "Gram stain" means the use of aqueous solutions of one of the methyl violet dyes, followed by the application of iodine in KI and differentiation by means of acetone, alcohol, aniline or aniline-xylene mixtures. The latter, which is employed in the eosin-Gram Weigert modification, is preferred by histologists on account of the slower differentiation which it allows. A variety of structures in tissue sections can be shown to be Gram-positive by the Weigert modification (Appendix 8, p. 828), some inconstantly, some constantly. Amongst these are fibrin, nuclear chromatin, hyaline droplets in the renal tubules, the heads of spermatozoa, mucin granules, collagen fibres and the β granules of the anterior pituitary gland. Between these structures and the Gram-positive bacteria there exists a quantitative difference at least, in that the latter have usually far greater resistance to decolorization by acetone or alcohol.

Theoretical Aspects of the Gram Stain

Older Theories

Bacteriologists have frequently endeavoured to find a satisfactory explanation for the mechanism of the Gram stain and many theories have been put forward, some chemical, some physical and some a combination of both. Schumacher (1926) believed that a crystal-violet-iodine-cell complex was formed in Gram-positive but not in Gram-negative cells, while Stearn and Stearn (1924) considered that the lower isoelectric point of the cytoplasm of the former cells, and their consequent stronger basophilia, was the main factor concerned. Iodine was relegated to the role of an oxidizing agent increasing the basophilia of the cytoplasmic proteins and thus their affinity for the basic dye methyl violet.

The factor of cell membrane permeability as responsible for the reaction has also been strongly supported. Burke (1922) and Burke and Barnes (1928, 1929) stated that the dye-iodine complex which was precipitated in both types of cell would pass readily only through the membrane of the Gram negatives; in this way they substantially agreed with the views of Benians (1920). Kaplan and Kaplan (1933)

also believed that the dye-iodine precipitate was formed in both Gram-positive and negative cells and that on subsequent treatment with alcohol it was dissociated and dissolved out of the latter. Other workers have regarded the Gram-positive property as due to the possession of a Gram positive layer of material coating an otherwise Gram-negative cytoplasm (Gutstein, 1925 ; Churchman, 1927, 1929).

The Hypothesis of Henry and Stacey

Strong evidence in favour of the views of Gutstein and Churchman was produced by Henry and Stacey (1943), who found that they could render Gram-positive organisms Gram-negative by a variety of methods. Chief of these was the extraction from the washed cells of a material, shown to be magnesium ribonucleate, by treatment with bile salts. There was no evidence, however, to suggest that solely an outer layer was affected. The Gram-negative cytoskeleton which remained could be restored to its original condition by recombination with ribonucleate, provided it was maintained in a reduced condition with formaldehyde or some other mild reducing agent. On the basis of this work Henry and Stacey suggested that the dye-retaining material in Gram-positive organisms was a high molecular complex formed by the combination of a reduced basic protein with magnesium ribonucleate. Bartholomew and Umbreit (1944) removed what they regarded as the outer layer of Gram-positive organisms with ribonuclease, and supported the former authors' conception. They considered more fully the nature of the bond between the ribonucleate and the material of the cytoskeleton which acts only in its reduced form and suggested that sulphydryl groups might be responsible. Three points supported this reasoning ; first, the autoxidizable nature of SH groups, which would only react in reduced form, secondly the effect of formalin which is known to prevent oxidation of sulphydryls, and thirdly the essential action of iodine, which is also known to react with sulphydryls, in the performance of the Gram stain. Panijel (1950, 1951), after a long series of experiments concerning the role of ribonucleoprotein in the Gram reaction, concluded that the Gram-positive structure produced by the magnesium ribonucleate-protein complex could be reproduced by a certain molecular disposition of the protein in the absence of RNA.

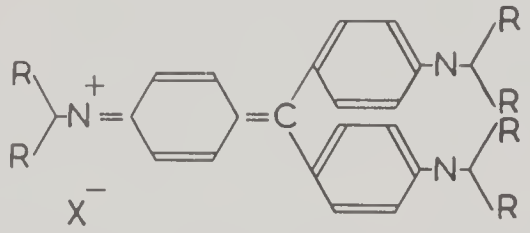
In later experimental work Henry, Stacey and Teece (1945) obtained from *Clostridium welchii*, and from yeast cells, nucleoproteins of an unusual type which when fixed on a slide were intensely Gram-positive. Material with these characteristics could not be obtained from Gram-negative organisms. The nucleic acid which they extracted was found to be entirely of the ribose variety while the protein, free from pentose and phosphorus, contained 5·5 per cent. of arginine and SS linkages

in addition. Neither the nucleic acid, nor the protein alone, were Gram-positive, but on reforming the complex in the presence of magnesium ions and formaldehyde at pH 5·0, the Gram-positive property was restored. When typical nucleates were made by adding nucleic acids to various basic proteins some of these, particularly histone-deoxyribonucleate, were found to retain methyl violet in some measure, but none so strongly as the natural substance from Gram-positive organisms. The authors considered that in the natural nucleoprotein the linkage of protein to nucleic acid was not of the simple electrovalent type, and that the mechanism of dye retention was more fundamental than its mere combination with a basophilic salt-like linkage. This work suggested that the cell membranes had nothing to do with the presence or absence of Gram-positive staining in bacteria, but that the latter depended on the unusual chemical or physical nature of the nucleoprotein present in organisms which reacted positively. Although Henry and Stacey (1943) had been able to " replate " both intact and crushed bacterial cells with magnesium ribonucleate, Mittwer *et al.* (1950) could not repeat this work on crushed cells and they therefore considered that the intact cell membrane was still an important factor.

Larose and Fischer (1953) examined the phenomenon of reversal of Gram staining in bacteria. They considered that the mechanism was similar to that previously found in wool fibres (Fischer and Larose, 1952a) which was thought to be due to oxidation of SH groups to SS groups. Larose and Fischer explained Bartholomew and Mittwer's finding that UV irradiation would restore Gram positive staining as due to reduction of SS to SH and SOH. Libenson and McIlroy (1955), however, disagreed entirely with this hypothesis and stated that SH groups did not take part in the Gram reaction of bacteria. Mitchell and Moyle (1950) showed that while gelatin was Gram-negative formaldehyde-treated gelatin was strongly Gram-positive. Later (1954) they produced an entirely different hypothesis after finding that RNA and DNA levels in the two groups of bacteria (Gram-positive and Gram-negative) did not differ significantly while Gram-positive organisms contained larger (x2) amounts of lipid phosphate and organic phosphate. They suggested that the Gram reaction was due to the presence of a lipid polyglycerophosphate-protein in the cell envelope. Shugar and Baranowska (1957) were able to make Gram-positive the " peripheries " of different organisms by treatment with protein. If the organisms were then ruptured, these " peripheries " retained their staining properties. These authors concluded that the extraction of a substance from bacteria with loss of Gram-positive staining could not be accepted as proof of causation. With this view, at least, one is able to agree whole heartedly.

The Role of Dyes in the Gram Reaction

Before we consider the application of the knowledge gained from this experimental work on bacteria to the histochemical use of the Gram reaction, there remains to be discussed some recent evidence concerning the role of the dyestuff used and the mechanism of its combination with iodine. Bartholomew and Mittwer (1950) tested a large number of dyes for suitability in the Gram stain. They found that any dye which gave a precipitate with iodine would give differentiation between Gram-positive and Gram-negative organisms, and of the eight dyes which did so in a satisfactory manner, seven are chlorides or acid sulphates of basic triphenylmethanes with the general formula :



Here R may be H, NH_2, CH_3 or C_2H_5. The seven dyes are brilliant green and malachite green (diamino-triphenyl methanes), crystal violet, methyl violet B, ethyl violet, Hoffmann's violet, and basic fuchsin. The eighth dye, Victoria blue R, is a diphenylnaphthylmethane. It seems possible, therefore, that at one end of the dye molecule the basic groups react with the acid groups of the nucleoprotein, leaving the basic groups at the other end to react with iodine to form the protein-dye-iodine complex long ago conceived as being formed in the Gram reaction. There are other possibilities, however, and these will be referred to below. When treatment with the dye and later with aqueous iodine is followed by differentiation with alcohol or some other solvent, the protein-dye-iodine complex is dissociated and dye and iodine are extracted from all but Gram-positive organisms. In a later paper (the third in their series) Bartholomew and Mittwer (1951) reported on the ability of twenty-nine dye samples to replace crystal violet in the Gram procedure. None proved to be efficient substitutes. Nevertheless, it is important to note that Bartholomew *et al.* (1949) demonstrated that after the methyl esterification of acid carboxyl groups in bacterial cells, a Gram reaction could still take place if a suitable acid dye, such as acid fuchsin, was employed. Iodine forms a precipitate with acid fuchsin and is evidently able to do so after combination of the latter with methylated protein groups produced in the bacterial cell in the above procedure.

Iodine Substitutes

Investigating various substitutes for iodine in the Gram reaction, Mittwer *et al.* (1950) showed that bromine, picric acid and mercuric

iodide, all of which give dense precipitates with crystal violet, produced comparable results to those obtained by using iodine. A large number of reagents which gave equally good precipitates *in vitro*, however, would not allow performance of the Gram reaction. Mittwer *et al.*, therefore, considered that precipitate formation alone was not sufficient and that the critical factor was the relative insolubility of the dye-precipitates in alcohol or water, and presumably in other solvents. In addition to the factors of precipitate formation and the relative insolubility of these precipitates, a third factor was found. This was the permeability of iodine through the membrane of the bacterial cell. In aqueous solution iodine was found to permeate both Gram-positive and Gram-negative cells equally freely. In alcoholic solution, however, penetration into Gram-positive cells was very slow. Conversely, once deposited in the cells from aqueous solutions, iodine was relatively resistant to extraction from the Gram-positives by 95 per cent. alcohol, but easily removed by this solvent from the Gram-negatives. The above authors used heat-fixed bacterial smears for their experiments on permeability but did not discuss the influence, if any, of this factor.

Histological Application of the Gram Reaction

Transferring these findings to the Gram reaction as applied to tissue sections, it is at once obvious that in most of the structures listed earlier in the chapter as Gram-positive, ribonucleoprotein is unlikely to be present and the presence of a cell-membrane, in the usual sense of the word, can be excluded. We are left only with the formation of the dye-iodine precipitate, and its low solubility in various solvents, as essential factors for the histological performance of the Gram reaction. Obviously some other factor or factors must be present and recent experimental work, though it does not solve the problem, at least points the way to a better understanding. Foster and Wilson (1951) have shown that whereas the normal Gram reaction, considered by McLetchie (1944) to be specific for the purpose, stains only the β granules of the anterior hypophysis, an alcoholic solution of the crystal violet-iodine precipitate stains only the acidophil α granules. Thus conversion of the basic triphenylmethane dye into an acid dye by means of iodine results in complete alteration of its properties. Furthermore, I have observed that if Gomori's (1950) paraldehyde-basic fuchsin stain for elastic tissue (Appendix 7, p. 815) is followed by Gram's iodine and then by alcoholic differentiation, acidophil structures such as red cells become "Gram-positive." Since fuchsin can take the place of methyl violet in the true Gram reaction, this manœuvre is essentially a Gram reaction performed with a fuchsin whose chemical properties have been altered by combination with aldehyde. Although

Gomori did not explain the mechanism of his stain, it appears that the addition of aldehyde causes the basic dye basic fuchsin to behave to some extent as an acid dye with affinity especially for the NH_2 groups of the tissues. Paraldehyde is acetaldehyde trimer and in acid solution, as in Gomori's mixture, it breaks down slowly to yield acetaldehyde. This presumably forms a compound with the basic groups of fuchsin. Iodine is able to form a precipitate with the aldehyde-fuchsin complex *in vitro*, and it can then be shown that all the free aldehyde groups are present in the supernatant, the precipitate being composed of fuchsin and iodine only. When the reaction between aldehyde-fuchsin and iodine is performed on stained sections, subsequent differentiation with alcohol gives the apparent Gram effect in structures which previously stained with the aldehyde fuchsin. Since Bartholomew and Mittwer (1950) found that acid dyes, with the exception of acid fuchsin and aniline blue, gave no hint of Gram differentiation when used as substitutes for crystal violet, this paradoxical effect of aldehyde-fuchsin requires some explanation.

The positive Gram reaction of tissue components and cells has continued to absorb the interest of a number of groups of investigators. Most of the work reported has concerned keratin and its derivatives but Monné (1955 a and b) and Monné and Borg (1954) have examined the nature of the positive reaction given by the egg envelopes of a number of parasitic nematodes. The views of Fischer and Larose (1952b) on the role of SH groups in the Gram reaction of keratin have already been mentioned. Some authors (Harms, 1958) appear to agree with them while others (Johnson *et al.*, 1957) can find no evidence of the participation of SH. The abolition of the positive reaction by deamination, also observed by Panijel (1950) in the case of *Ascaris* sperm, certainly suggests that reactive protein groups other than SH are concerned but no other evidence, as far as I know, implicates the NH_2 groups in this way. Theoretically one would expect that the basic NH_2 groups would compete with the basic CH_3 groups of the dye for acid binding sites. The latter would then be released by deamination. Probably the nitroso or hydroxyl groups produced by deamination interfere in some way. Gianni (1952) and Gianni and Della Torre (1952) ascribe the positive Gram reaction to the presence of adjacent COOH groups on the same molecule (aspartic and glutamic acids) and their view agrees with that of Panijel (1951). The latter, maintaining that acid groups are essential, showed that those of the dicarboxylic amino-acids were sufficient. Monné, moreover, determined that the Gram-positive reaction of the fibrous protein of his helminth ova was due to COOH groups although he also implicates acid polysaccharides and phenolic substances.

To my mind, the only explanation which will fit the facts is that

iodine is able to displace the triphenylmethane dyes from combination with acid groups whatever their nature, reacting with some, or all, of the basic side chains to give the usual iodine-triphenylmethane precipitate. I therefore consider that in the histochemical performance of the Gram reaction the initial acidic protein-crystal violet complex is broken by iodine, which combines with the basic groups at both ends of the triphenylmethane molecule to form a relatively alcohol-insoluble crystal violet-iodine precipitate *in situ*. With subsequent differentiation this precipitate is less easily removed from certain structures than from others, two main factors being concerned. First, more of the crystal violet-iodine complex is formed where there was initially more crystal violet in combination with protein and secondly, certain physical barriers offer resistance to the extraction of crystal violet-iodine by alcoholic or other differentiation. Among the physical barriers may be mentioned the presence of a lipid or lipoprotein membrane, such as is probably present in Russell bodies, and the arrangement of protein molecules, having the necessary reactive acid groups, in the form of a close network. Such a state is often referred to as polymerized. In this context the similarity of results obtained by the coupled tetrazonium reaction after benzoylation (Chapters V and VII), and the Gram reaction using aniline-xylene differentiation, is particularly striking. Figs. 74 and 75 illustrate this point. The first shows intracellular mucin in the human stomach mucosa stained by the tetrazonium reaction after 16 hours benzoylation; the second shows the Gram reaction on similar material. Gastric and intestinal mucin granules, salivary gland mucoid granules, pituitary β granules, Russell bodies and collagen, to mention a representative few, are all stainable by either method, but the two methods do not give exactly parallel results, for two reasons. First, the coupled tetrazonium reaction does not depend on the presence of a sufficient number of reactive basophil (acid) groups, as does the Gram reaction. For this reason a variety of Gram-negative, acidophilic, structures are able to give a positive tetrazonium reaction when the physical state of their aromatic amino-acids (most probably histidine) affords protection from benzoylation. Secondly, from some structures such as reticulin, which are also positive by the tetrazonium reaction after benzoylation, the crystal violet-iodine complex is too easily removed for them to behave as Gram-positive. Mild heat, which has so interesting and profound an effect on the intensity with which various structures react by the tetrazonium method after benzoylation has decidedly less effect on the degree of Gram-positiveness manifested by tissue structures. In some cases, however, the effect is still quite noticeable.

In addition to the initial and insuperable objection to the use of the Gram stain as a histochemical reaction, because of the impossibility

of controlling differentiation adequately, further conditions must now be imposed. If the Gram reaction alone is used, and a particular tissue component is found to be Gram-positive, the only conclusions which can legitimately be drawn are: (1) that reactive acid groups are present, and (2) that either the constituent molecules are in some way condensed or polymerized, or else a physical barrier such as a lipid or lipoprotein membrane is present. Although this information may be useful by itself, further confirmation must be obtained by more histochemically-reliable methods if the Gram reaction is to achieve its full degree of usefulness.

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CHAPTER IX

CARBOHYDRATES

A Classification of Carbohydrates Identifiable in Sections

I. Polysaccharides

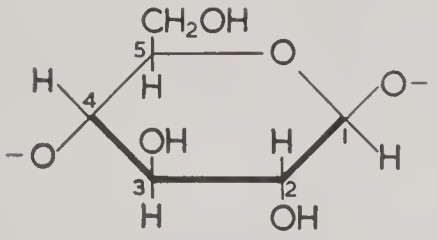
GLYCOGEN is the only naturally-occurring member of this group remaining in animal tissues after aqueous fixation and paraffin embedding. It is also found in a number of different microorganisms and in the plant kingdom, but only in the primitive thallophytes. There are a number of different glycogens of varying degrees of polymerization. All are composed of chains of α-glucopyranose (D-glucose) units, linked α-1,4′ except at points of branching where the linkage is α-1,6′, and they are readily hydrolysed to glucose by boiling with dilute acids. The solubility of glycogen in various fixatives has been much exaggerated and Baker (1945) stated that formalin fixation preserved proteins in such a way that glycogen was held by them and not easily dissolved by water. Picric acid has a similar effect. Vallance-Owen (1948) found formalin as effective as absolute alcohol in fixing glycogen and observed no diminution in the quantity of this substance after leaving tissue for 38 days in the former fixative, and after 12 hours subsequently in running water. The insolubility of fixed glycogen is probably at least partly due to its precipitation in the form of a protein complex, since in the native state glycogens are probably bound to albumins by secondary valency bonding (van der Waal's forces).

STARCH, in various forms, is the main carbohydrate reserve substance of the plants. On hydrolysis the starches yield only D-glucose but they are composed of two different glucans, one a linear molecule called amylose and the other a branched molecule called amylopectin. The amylose-amylopectin ratio varies from case to case. The straight chain maltose polymer (amylose) consists of about 20 glucose residues joined α-1,4′, while the branched chains consist of 24 to 30 glucose residues with an α-1,6′ linkage joining one chain to another.

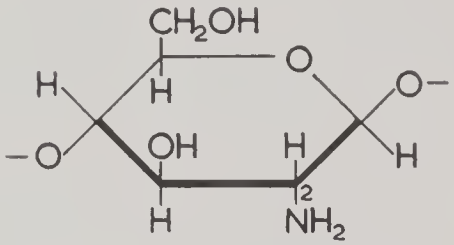
CELLULOSE is the main constituent of the cell walls of land plants and, as such, it is more plentifully distributed than any of the other polysaccharides. Like the two preceding polysaccharides cellulose yields only glucose on total hydrolysis. It is regarded as a long chain of D-glucopyranose units connected uniformly by β-1,4′ linkages except in certain regions where so-called weak linkages occur. The latter are easily hydrolysed with the setting free of the well recognized long chain polymer.

II. Mucopolysaccharides

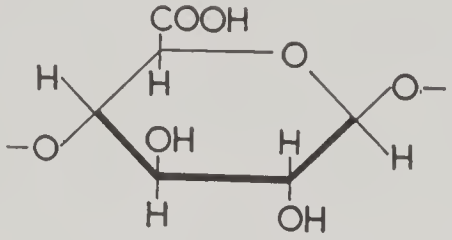
These were defined by Meyer (1938) as polysaccharides containing hexosamine as one component, and occurring either free or as esters of sulphuric acid. The esters usually occur in loose combination with protein substances of higher molecular weight from which, in the case of acid mucopolysaccharides, they may be obtained in the free form by mild alkaline hydrolysis. The acid mucopolysaccharides, both simple and complex, contain hexuronic acid as their second carbohydrate component, while the neutral mucopolysaccharides contain hexoses instead. The modern structural formulæ of their carbohydrate components, shown as radicals, follow:



β D-Glucose



β D-Glucosamine



β D-Glucuronic acid

Meyer considers that neutral mucopolysaccharides from animal sources always occur in firm combination with protein, and a small fraction of gastric mucus, in the pig, was found to be a neutral mucopolysaccharide by Meyer, Smyth and Palmer (1937). The acid mucopolysaccharides are widely distributed; they are components, as a rule, of all epithelial mucins of the alimentary canal, and they occur in the majority of duct mucins also.

Chitin is the simplest neutral mucopolysaccharide, consisting only of *N*-acetylglucosamine residues joined together in pairs by β-1,4, glucosidic linkages. It is thus essentially cellulose in which the hydroxyl groups on the C2 carbon atoms are replaced by $-NHCOCH_3$. In the plant kingdom it is restricted to certain of the lower plants but in the invertebrates it is the principal structural component of the exoskeleton or shell, hence its name χιτών, a tunic or coat of mail. It is most abundant in arthropods, annelids and molluscs and also in the insects. Pure chitin, however, does not occur in nature ; it is always mixed with calcium carbonate, or protein, or both.

Other neutral mucopolysaccharides are found in pig gastric mucosa and in the capsules of *Pneumococcus*. These contain acetylglucosamine

and galactose. The polysaccharide of *Shigella dysenteriae* on hydrolysis yields acetylglucosamine, galactose and rhamnose.

Hyaluronic Acid is a simple acid mucopolysaccharide, composed of *N*-acetyl-D-glucosamine and D-glucuronic acid units, which occurs probably as a freely dissociated compound in vitreous humour and in synovial fluids. It occurs also in complex form as hyaluronosulphate.

Heparin is another complex mucopolysaccharide composed of glucosamine, glucuronic acid and sulphuric acid.

Chondroitin Sulphuric Acid is one of the main polysaccharide components of mammalian connective tissues and of cartilage. There are still differences of opinion as to its constitution but the view of Davidson and Meyer (1955) is usually accepted. These authors regard the repeating unit as a disaccharide composed of sulphated *N*-acetylgalactosamine joined to glucuronic acid by a β-1,3′ glycosidic linkage.

III. Mucoproteins and Glycoproteins

Mucoproteins (mucoids) are defined by Meyer as substances in which hexosamine-containing polysaccharide is found in firm chemical union with a peptide, the hexosamine content of the whole being greater than 4 per cent. Hexose and hexosamine are the usual carbohydrate components of mucoproteins, though uronic acids occur in some examples.

Glycoproteins are distinguished from mucoproteins on the arbitrary but convenient basis of their hexosamine content of less than 4 per cent. This division, introduced by Meyer, is of service to biochemists and it may usefully be followed in histochemistry, although only rough histochemical distinction is at present possible. The mucoproteins include submaxillary gland mucoid, the serum mucoproteins seromucoid and seroglycoid, and the larger fraction of gastric mucus in the pig (Meyer and Smyth, 1937). Chorionic gonadotrophin in all species so far examined, and the precursor substances of the pituitary gonadotrophins (FSH and LH), and thyrotrophin, are also mucoproteins. Fractions of both serum albumin and serum globulin have been described which fall into the group of glycoproteins: thus Blix *et al.* (1941) isolated two fractions of human serum albumin containing 1·1 and 1·2 per cent. of hexose, respectively, and 0·45 and 0·56 per cent. of hexosamine. Tiselius (1937) gave the following figures, for the hexose content alone of normal horse serum globulins: α globulin 0·4 per cent., β globulin 0·2 per cent., and γ globulin 0·7 per cent. If the characteristic "mucoid" hexose-hexosamine ratio of 2 to 1, or even the less characteristic 1 to 1 ratio, is maintained in these fractions they can be classed as glycoproteins. There is apparently no lower limit for hexosamine content which separates glycoproteins from simple proteins. According to Meyer (1945) simple proteins containing hexoses without hexosamine have not been found in nature.

IV. Glycolipids

The cerebrosides, represented chiefly by phrenosin and kerasin, are the principal members of this group. They occur in tissues outside as well as within the central nervous system. These substances contain no phosphoric acid but yield on hydrolysis one molecule of fatty acid, one of sphingosine,

$$CH_3(CH_2)_{12}.CH : CH.CH(NH_2).CH(OH).CH_2OH$$

and one of sugar. The latter is usually galactose but glycolipids containing glucose do occur, as in the glucosidocerebrosides of the abnormal " kerasin " of Gaucher's disease (Ottenstein, Schmidt and Thannhauser, 1948). The inositol-containing lipids, mentioned by Hotchkiss (1948), are conveniently though improperly considered with this group. Among them are the phosphoinositides (Folch and Sperry, 1948), of which four or five members are known. One of these, diphosphoinositide, occurs in the kephalin fraction of brain tissue. According to Malaprade (1934) polyalcohols are oxidized by periodic acid at the terminal CHOH groups in the chain to form aldehydes which can react with Schiff's reagent. Inositol derivatives might react in this manner but inositol itself, having no terminal CHOH group, is converted into formic acid. Diphosphoinositide, isolated by Folch and Sperry, was also found to react with two molecules of periodic acid to yield one molecule of formic acid.

V. Ascorbic Acid

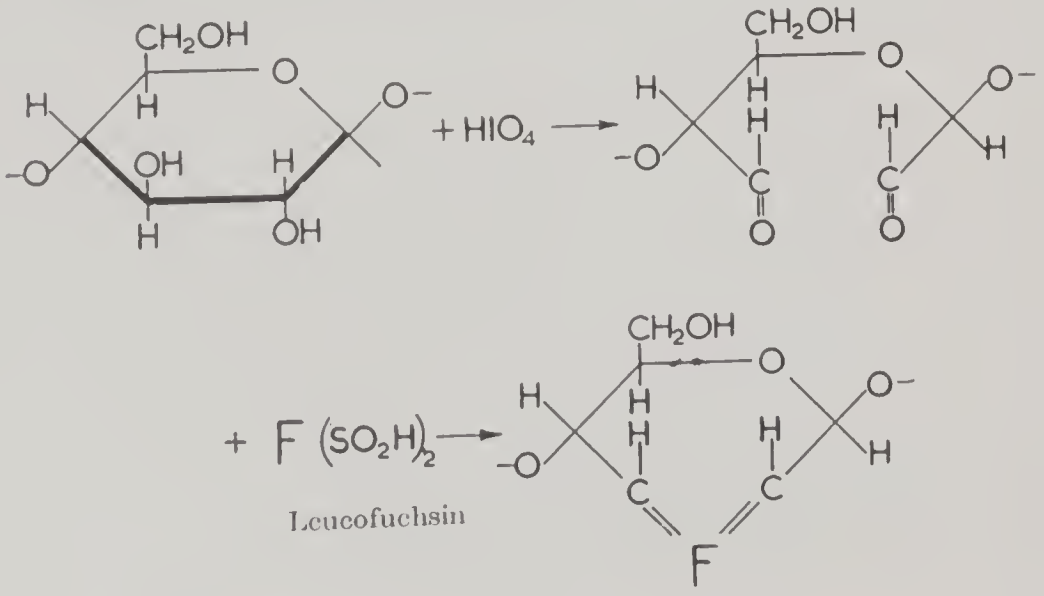
Although vitamin C fits into the classification of carbohydrate substances identifiable in the tissues, and it is therefore considered at this point, it properly belongs to a separate section altogether. All the other carbohydrates in the list are identifiable by reactions involving several properties. Ascorbic acid is localized solely on account of its function as a strong reducing agent. It is regarded as a derivative of the hexose L-gulofuranose.

Principles of the Periodic Acid-Schiff Reaction

The whole of the modern histochemistry of the polysaccharides, mucopolysaccharides and mucoproteins is bound up with the periodic acid-Schiff reaction (PAS), and it is therefore necessary to consider at this point the principles on which this reaction is based. Periodic acid (HIO_4) is an oxidizing agent which was employed by Malaprade (1928, 1934) for the chemical estimation of glycols ($CH_2OH.CHOH$) and by Nicolet and Shinn (1939, 1941) for amino-substituted alcohols ($CH_2OH.CHNH_2$) such as those which occur in the amino-acids serine, threonine and hydroxylysine. It was first employed as a reagent for polysaccharides by Jackson and Hudson (1937). Its use in histology

was first described by McManus (1946) for the demonstration of mucin, but later Lillie (1947a and b) and Hotchkiss (1948) elaborated the method into a histochemical one which could be used to demonstrate a variety of polysaccharide substances in the tissues. McManus (1948a) regarded the method as primarily of histological importance but later (1948b) accepted Hotchkiss's view that it could be used legitimately in histochemistry. The importance of the method in carbohydrate histochemistry is emphasized by the number of papers describing work in which it has been employed, often as the sole reaction, and by a number of excellent reviews on the subject which should be consulted by those particularly interested in the matter (Hashim, 1952; Gedigk, 1952; Graumann, 1952, 1956; Bangle and Alford, 1954; McManus, 1954; Lillie, 1953, 1954; Hale, 1957; Leblond *et al.*, 1957).

Periodic acid is an oxidant which breaks the C—C bonds in various structures, where these are present as 1 : 2-glycol groups (CHOH—CHOH), converting them into dialdehydes (CHO.CHO). The equivalent amino or alkylamino derivatives of 1 : 2-glycol or its oxidation product (CHOH.CO) are also attacked and converted into dialdehydes. The particular property of periodic acid, which renders it immeasurably superior to other reagents commonly used in histochemistry for oxidation of C—C bonds ($KMnO_4$, H_2CrO_4, H_2O_2), is that it does not further oxidize the resulting aldehydes and these can, therefore, be localized by combination with Schiff's reagent to give a substituted dye which is red in colour. Carbonyl groups are said to be oxidized to carboxylic groups and thereby prevented from subsequent reaction with Schiff's reagent. The reaction of periodic acid with the dehydroglucose residue of a polysaccharide molecule is illustrated below. It should be noted that the red dyestuff formed by union of fuchsin-sulphurous acid with dialdehyde is a new compound and not, as previously supposed, re-oxidized fuchsin.



The amount of colour developed by the reaction is dependent on the amount of reactive glycol structure present in the tissues and Glegg *et al.* (1952) consider that the reactive groups concerned are those of the hexose sugars glucose, galactose and mannose and of the methylpentose sugar fucose. This view ignores the possible part played by hexosamines and hexuronic acids but Hooghwinkel and Smits (1957) found that the hexosamine groups of CSA and hyaluronic acid were intact after oxidation with periodic acid. They noted at the same time that although the hexuronic acids were oxidized no aldehydes could be demonstrated. It was therefore concluded that " the substances responsible for a positive PAS reaction must be entirely or partly built up of other carbohydrates than hexosamine and hexuronic acid."

According to Hotchkiss (1948), positive results should be given by any substance which fulfils the following four criteria:

(*a*) contains the 1 : 2-glycol grouping or the equivalent amino or alkylamino derivative, or the oxidation product CHOH—CO;
(*b*) does not diffuse away in the course of tissue fixation ;
(*c*) gives an oxidation product which is not diffusible ;
(*d*) is present in sufficient concentration to give a detectable final colour.

A large number of substances contain the 1 : 2-glycol grouping for which the reaction is specific and should give a positive result, **but compounds in which the glycol groups are substituted do not do so.** In the latter class are ribonucleic acid, in which the glycol group is substituted by phosphoric acid, and serine, threonine and hydroxylysine when these are combined in the polypeptide chains of proteins. Hotchkiss stated that those naturally-occurring animal substances which gave a positive result in practice were monosaccharides, polysaccharides, mucoproteins, glycoproteins, phosphorylated sugars, cerebrosides and inositol-containing lipids. He considered that after the use of ordinary aqueous fixatives only substances of high molecular weight remained in the tissues in sufficient quantity to give a positive result. Such substances were polysaccharides, hyaluronic acid, mucoproteins and mucins.

Since the first edition of this book was written, largely due to the work of Leblond and his associates (Glegg *et al.*, 1952; Glegg, Eidinger and Leblond, 1953, 1954) the status of the PAS reaction in histochemistry has been greatly clarified. In Table 14 I have listed, in various groups, the types of material which may be expected to give a positive PAS reaction. In this table there is no reference to the type of preparation employed (fresh sections, formalin-fixed frozen sections, paraffin sections, etc.) but the individual components listed therein will react positively if they are present in concentrations above a certain mini-

TABLE 14

PAS-positive Tissue Components

I	Polysaccharides		Glycogens Starches Cellulose
II	Neutral Mucopolysaccharides	Bacterial capsular polysaccharides	Neutral m.p.s. of gastric mucin
III	Muco- and glyco-proteins	Salivary gland mucoid Seromucoid, seroglycoid Gastric mucoid Chorionic gonadotrophin Pituitary gonadotrophins (FSH, LH). Pituitary TSH Blood group A substance Thyroglobulin	Fractions of : serum albumin serum globulin reticulin collagen
IV	Glycolipids	Gangliosides Cerebrosides { Phrenosin / Kerasin	Inositol phosphatides
V	Unsaturated lipids and phospholipids	Sphingomyelin	Lipofuscins Cardiolipin Ceroid

mum level, and if the degree of oxidation employed has been adequate (see below).

Four of the five groups which give a positive reaction with PAS have already been defined in the classification of carbohydrates. The fifth group, which contains under the heading of lipids and phospholipids a number of non-carbohydrate-containing substances, is important enough in relation to the PAS reaction to be considered here in this chapter as well as in Chapter XI. The main substance in the phospholipid group is sphingomyelin, whose final hydrolysis products are sphingosine, fatty acids, phosphoric acid and choline. Since sphingosine contains a primary acylated amine adjacent to a hydroxyl group it is capable of reacting with HIO_4 to give an aldehyde and thus a positive PAS reaction. Verne (1929) showed that many " pure " fats, among them triolein, butter fat, and lecithin, would give a typical aldehyde reaction with Schiff's solution after mild oxidation. Evidence on the production of aldehydes from lipids and phospholipids by periodic acid oxidation of tissue sections cannot yet be considered conclusive, but Hack (1949) has shown that the following lipid substances, *in vitro*, react with Schiff's reagent after oxidation with periodic acid : kerasin and phrenosin (cerebrosides) phosphatidylethanolamine and phosphatidylcholine, acetal and inositol phosphatides, and ganglioside. Obviously we require to know much more about the reaction of periodic acid with lipids other than glycolipids. Wolman (1950) carried out

some useful investigations in this respect. By means of a Coujard type of technique (see Chapter V, p. 112), this author studied the effect of oxidation by HIO_4, $KMnO_4$ and Cr_2O_3 on pure samples of cerebroside and sphingomyelin, and on a lecithin-kephalin mixture. He also used commercial samples of saturated and unsaturated fatty acids. The effect of oxidation was revealed by 10 minutes' treatment with Schiff's reagent. Wolman concluded that unsaturated lipids stained while saturated lipids did not ; sphingolipids stained positively whether they contained a carbohydrate moiety or not. Later (1956) Wolman confirmed some of these results and showed that the positive reaction of sphingomyelin was dependent on the strength and duration of oxidation. If a 1 per cent. solution of HIO_4 was employed, and an oxidation time of less than ten minutes, practically no staining occurred.

From the practical point of view, therefore, there is no doubt that lipid substances in the tissues, present as free lipids or as lipo-protein complexes, which can reasonably be considered to contain no glycolipid, can react positively by the PAS technique.

In paraffin sections, however, they are either no longer present or present in such small quantities that they do not react. Leblond *et al.* (1957) consider that once glycogen has been removed, only one class of materials gives a positive PAS reaction. These are referred to as carbohydrate-protein complexes and they include the neutral mucopolysaccharides (in part) and the muco- and glycoproteins of the classification employed in this chapter. Glegg *et al.* (1954) found that these complexes contained, as their main carbohydrate components, the hexose sugars glucose, mannose and galactose, together with the methylpentose sugar fucose, and various hexosamines. The intensity of PAS staining in paraffin sections has been found by Leblond *et al.* (1957) to correspond closely with the values for hexose sugars found in tissue extracts and they stress the fact that their Fraction I of these extracts, which contained all the acid mucopolysaccharides present in the original, was PAS-negative. Fraction II, containing the above-mentioned hexoses, fucose, hexosamines and sialic acid, was strongly PAS-positive.

It is clear, from the work of these and other authors that the acid mucopolysaccharides, assumed by myself and others to be capable of giving a positive though weak reaction with PAS, in fact do not react at all. The weak PAS reaction of intensely metachromatic tissue components containing acid mucopolysaccharides is thus due to carbohydrate-protein complexes of the type mentioned above. The *in vitro* tests carried out by Braden (1955) on various acid mucopolysaccharides confirm this impression, as do those of Hooghwinkel and Smits (1957) who find that both CSA and hyaluronic acid are PAS-negative.

Table 15

Identification of Carbohydrate-containing materials

Group	Material	Millon or tetrazonium	SSB †*	Alkaline tetrazolium	PAS	Diastase	Metachr. (gamma)	Metachr. (beta)	MBE	Alcian blue (ph 2)	Hale	Hyaluronidase
I & IA	Glycogen . .	—	—	—	+	Labile	—	—	*c.* pH 6	—	—	Fast
IB	Starch, cellulose .	—	—	+ or —	+	Fast	—	—	. .	—	—	Fast
IIA	Chitin . . .	+ §	—	+	—	Fast	—	—	Above pH 4	—	—	Fast
IIB	Acid mucopolysaccharide (Epith. mucin)	+	—	+	—	Fast	+	—	Below pH 4	+	+	Usually fast
IIB	Hyalurosulphate or CSA ‡ Type A	+ or § weak	—	+	—	Fast	+	—	Below pH4	+	+	Labile
IIB	CSA ‡ Type B (aorta, skin)	+ §	—	+	—	Fast	+	—	Below pH 4	+	+	Fast
IIB	CSA ‡ Type C (umbilical cord)	+ §	—	+	—	Fast	+	—	Below pH 4	+	+	Labile
III	Mucoprotein or glycoprotein	+	—	+	+	Fast	—	+ or —	Above pH 4	Faint or —	—	Fast
. .	Simple basic protein	+	—		—	Fast	—	—	*c.* pH 6	—	—	Fast
IV	Glycolipids . .	—	+	+	+	Fast	—	—	Often pH 4	—	—	Fast
V	Sphingolipids .	+ §	+	—	+ † or —	Fast	+ † or —	—	Varies	—	—	Fast

* Sudan black B (preferably on formol-fixed frozen sections as well).
† Depending on method of preparation of sections.
‡ Chondroitin sulphuric acid.
§ If present as protein complexes.

Identification of Carbohydrate Substances (except Ascorbic Acid)

Table 15 is intended to indicate the way in which the various groups in the above classification can be distinguished one from another, but it must be understood that distinction is not possible between mucoprotein and neutral mucopolysaccharide by these methods. Moreover, our difficulties are increased by the frequent, one might almost say invariable, occurrence of mixtures of the mucins.

Identification of protein by either the Millon or the coupled tetrazonium reaction has been discussed in Chapter V and the use of Sudan black B is dealt with in Chapter XI. Diastase and hyaluronidase are fully considered in the chapter (XXII) on the use of enzymes as histochemical reagents. The other reactions in the table are considered in this chapter, below.

Group I

All the methods for diagnosing and demonstrating the presence of glycogen are to be found towards the end of the chapter (p. 265) under the heading "Methods for glycogen." No difficulty is usually experienced in separating this material from the other material in the table.

Group IIA

Chitin, which is normally PAS-negative (cf. Brunet, 1952) may become positive after deacetylation. It reduces the alkaline tetrazolium reagent to give a strong blue colour. (See Appendix 9, p. 832.)

Group IIB

The epithelial and connective tissue mucins in this group are distinguished from substances in Groups IIA and III by the strong (gamma) metachromasia which they give under suitable conditions. They tend to show a low pH value for methylene blue extinction, but this test is limited by the fact that in certain regions, such as in goblet cells, affinity for methylene blue is extremely small even at alkaline pH levels.

Group III

The mucoproteins and glycoproteins were subdivided according to Meyer's arbitrary but convenient classification and no real histochemical distinction is possible between them. Nor at present can they be distinguished from neutral mucopolysaccharides of the type occurring in gastric mucins. In practice, substances which contain less than about 0·5 per cent. of polysaccharide, calculated on a dry weight basis, fail to give any recognizable colour with fuchsin-sulphurous acid after periodic acid if this reaction is carried out as suggested in Appendix 9. I have calculated, from figures given by Seibert *et al.* (1948) for the

albumin and globulin fractions of normal human serum, to which figures for fibrinogen have been added, that normal plasma contains about 1·4 per cent. polysaccharide (dry weight). The colour given by formalin-fixed plasma, from which lipids have been extracted, after treatment with diastase and staining by the PAS method is a recognizable but faint pink. This agrees fairly well with the observations of Leblond *et al.* (1957) that the **hexose** values can be correlated with intensity of PAS staining. These authors found that rat seminal vesicle secretion (0·7 per cent.) stained very faintly while the contents of the coagulating gland (11·0 per cent.) were brilliantly stained. Mucoproteins therefore stain more strongly than glycoproteins. **These two materials have not been found to give strong (gamma) metachromasia so that the presence of such metachromasia can be taken to rule out substances in Group III as responsible for the effect.** Protein materials in the tissues, which either contain no lipid or from which lipid has been extracted (see Chapter XI), which contain diastase-fast carbohydrate in sufficient quantity to give a colour by the PAS method, but which neither exhibit gamma metachromasia with toluidine blue nor bind methylene blue below pH 4, are to be regarded as muco- or glycoproteins, or as carbohydrate-protein complexes. The presence of a positive Millon reaction for tyrosine or a positive coupled tetrazonium reaction for tyrosine, tryptophan and histidine, can be taken as sufficient indication of the presence of protein.

Groups IV and V

The possibility has to be considered that PAS-positive material in paraffin sections may belong to these groups. Although Hotchkiss claimed that cerebrosides and inositides were removed from paraffin sections both in the process of embedding and in passage through the 70 per cent. alcoholic solutions which he employed, there is good evidence that some are too firmly fixed, after the use of formalin, for this to occur. Morrison and Hack (1949) obtained a "brilliant" colour, using the PAS method on formol-fixed paraffin sections from a case of Gaucher's disease, in the lipid-filled cells characteristic of the disorder. In a single case of this disorder I found only a moderate reaction in the specific lipid when tested with the PAS method, but this may have been due to such extraction of lipids as is known to occur with prolonged sojourn in formalin. The structure of the kerasin molecule would not lead one to expect a strongly positive result ; it possesses only one reacting group per molecule and carries two very long alkyl side chains. By comparison, the much smaller repeating unit of mucoitin sulphuric acid has four reacting groups, closely spaced together. There is no doubt, however, that substances in Group IV (cerebrosides and gangliosides), present in fixed tissues,

give a positive PAS reaction. In paraffin sections of normal, as opposed to pathological, tissues cerebrosides and gangliosides are seldom much in evidence. In gargoylism and in the amaurotic idiocies, however, the latter appear in brain and peripheral tissues in considerable quantities. They are brilliantly PAS-positive. Substances in group V have not been shown to give positive PAS reactions in paraffin sections unless unusual conditions of oxidation are employed. If any doubt remains distinction must be made between lipids and non-lipid groups, as responsible for the PAS reaction, and this can be done in various ways. If the material under consideration is not limited in amount, further blocks should be fixed in hot acetone or other lipid extractive, or in weak Bouin followed by pyridine extraction of the sections (Baker, 1946). After such procedures, which effectively remove triglycerides, phosphatides and cerebrosides, any material which still gives a positive PAS reaction must be in Groups I, II or III. If, on the other hand, only formalin fixed tissue is available, control paraffin and preferably also frozen sections should be stained with Sudan black B. This dye, in contrast to the red Sudan dyes, stains phospho- and glycolipids as well as neutral fats. In any PAS-positive structure shown to contain lipid by this procedure, the responsibility of such lipid for the reaction cannot be excluded unless control sections, suitably fixed and freed from lipid by one or other of the above types of extraction, have been similarly stained. Lipids are not wholly extractable from materials fixed in formalin. If the PFAS or PAAS reactions (Appendix 11, p. 859) give a positive result in a given structure it must be assumed (in the absence of keratin) that unsaturated lipids are present. The part played by these, in their normal (unoxidized) form, in producing a positive PAS reaction is certainly very small, perhaps non-existent.

Individual Methods for Carbohydrate-containing Substances

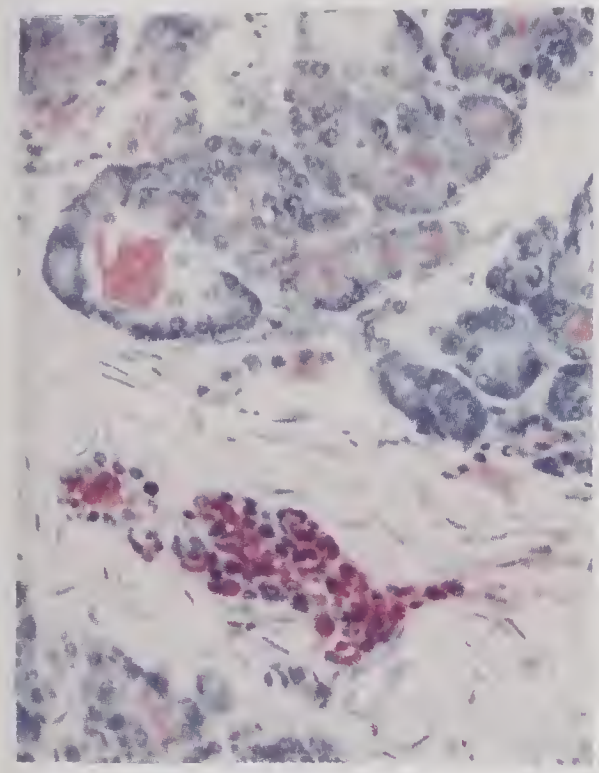
Further Considerations of the PAS Reaction

Oxidation. In carrying out the PAS technique it is usual to employ the double hydrate of periodic acid (H_5IO_6) which is easily obtained from most manufacturers of organic chemicals. Lillie (1947b) employed a modification in which periodic acid was produced from 1 per cent. sodium periodate (Na_3IO_5) by the action of dilute nitric acid (0·5 ml. of 70 per cent. acid per 100 ml.). This method has the advantage that a fresh periodic acid solution is produced from the stable periodate at the time of use, but it is less useful now that periodic acid of reputable quality is available. Within wide limits (0·5 to 2·5 per cent. HIO_4) the strength of the solution of periodic acid makes no difference to the final result.

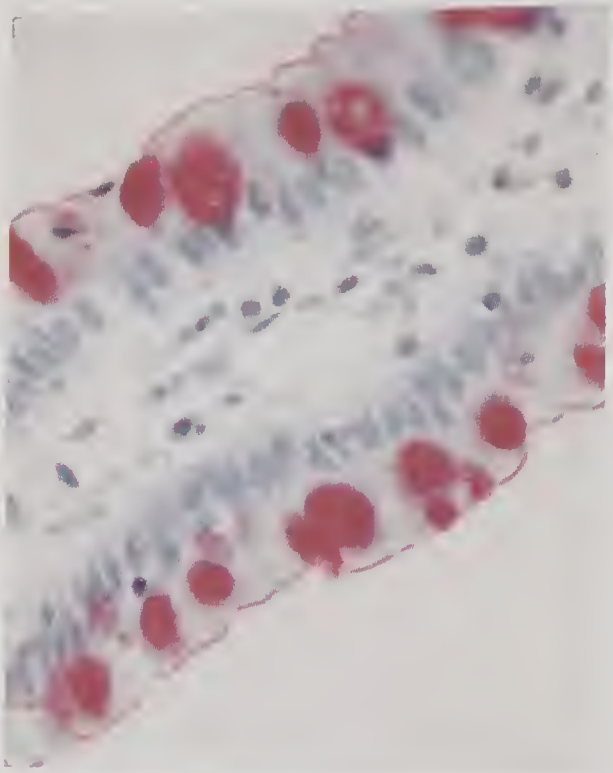
Solvent. Only two solvents have so far been used for periodic

acid in histochemistry ; they are water and ethyl alcohol. A 70 per cent. solution of the latter, buffered with M/5 sodium acetate, was recommended by Hotchkiss (1948) for use whenever there was any question of the solubility in water of the polysaccharide under investigation, or of its oxidation product. He suggested that, where such precautions were judged unnecessary, aqueous solutions, which are more rapid and vigorous in action, might be used instead. At the present time the buffered alcoholic solution of Hotchkiss is infrequently employed. Even for staining glycogen (q.v.), for which it was originally intended, it has been found to give far less staining than the conventional 0·5 per cent. aqueous solution, after treatment with Schiff's reagent. For staining the very water soluble dextrans Mowry (1952, Mowry and Millican, 1952) has used periodic acid in 90 per cent. ethanol. With this combination the time of oxidation must be extended to 120 minutes or more.

Time of Reaction. The time of exposure of sections to the acid is especially important. Dempsey, Singer and Wislocki (1950) showed that exposure to 1 per cent. aqueous periodic acid for 1 hour at 37° resulted in a great increase of tissue basophilia as judged by the depth of methylene blue staining. The ability of the oxidized tissues to stain with this dye at low pH values indicated that the reactive groups dissociated as strongly as some mineral acids, and the authors therefore suggested that the increased basophilia might be due to the formation of sulphonic acids by oxidation of tissue sulphydryl groups. Support for this view was derived from the structures in which the increased basophilia was especially manifest, including epidermis, hair shafts and follicles, and the endothelia of blood vessels. If Hotchkiss is correct in his assertion that periodic acid has a negligible tendency to oxidize aldehydes to carboxylic acids, and if it can be shown that the latter are not sufficiently strongly dissociated to combine with methylene blue at low pH values, then the assumption of Dempsey *et al.* that sulphonic acids are responsible for the increased tissue basophilia after periodic acid oxidation is probably correct. However, as these authors admit, the increased basophilia produced by oxidation with potassium permanganate is comparable with that produced by HIO_4 and this similarity can be shown to exist whether methylene blue, or Schiff's solution, is used as the final indicator of the reaction products. Permanganate is known to break some bonds between adjacent carbon atoms and to oxidize these progressively to carboxylic acids *via* carbonyl groups, but this action is not restricted to 1 : 2-glycols as it is stated to be with periodic acid. Lillie (1951) made a comparison of the various types of oxidation used in histochemistry with Schiff's solution as final indicator. The differences were in some cases profound. Lhotka (1952a and b) tested the effect of different

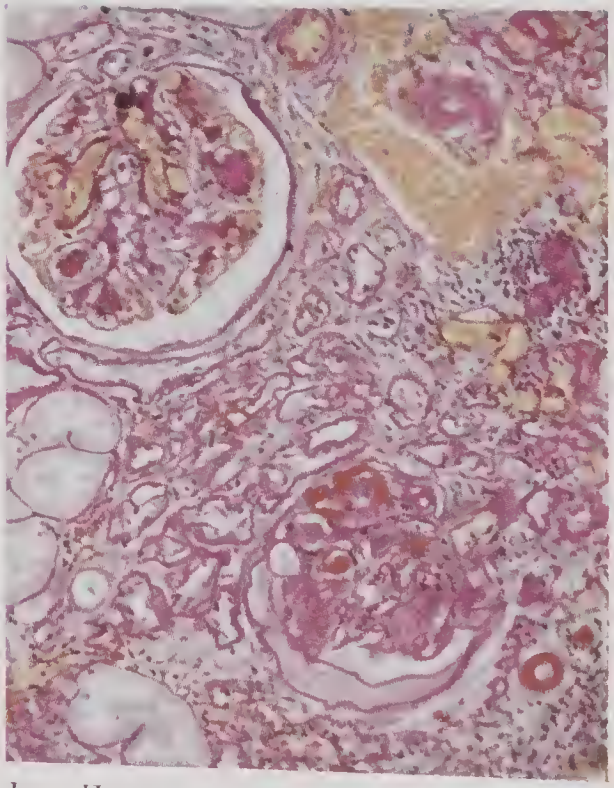


Ia. Human pancreas, freeze-dried formalin-alcohol-fixed section. Periodic acid—Schiff, toluidine blue. × 525.



Ib. Human duodenal villus (cf. Plate Ic). Strongly PAS-positive mucin in the goblet cells contrasts with less positive mucin in the brush border. Periodic acid—Schiff, celestin blue, hæmalum. × 440.

Ic. Human duodenal villus (cf. Plate Ib). Sixteen hours' benzoylation at 22°. Coupled tetrazonium reaction. × 440.



Id. Human kidney. Kimmelstiel-Wilson syndrome. (Overstained to provide colour contrast.) Trichrome-PAS method. × 97.

[*To face p.* 240.

times (and temperatures) of oxidation. He claimed that whereas the standard 2 minutes' exposure to periodic acid would oxidize *cis*-glycols readily *trans*-glycols would be affected much more slowly. Using gelatin blocks containing isomers of cyclohexane (1952c) Lhotka found that *cis*-glycols would react with Schiff's reagent after 10 seconds oxidation and that the reaction was complete after 60 seconds. The *trans*-glycols gave a colour after 30 seconds and oxidation was complete after 2 minutes. In practice these differences have not proved sufficient basis for distinction between the two types of glycols by the PAS method. McManus (1956) maintains that 0·5 per cent. aqueous HIO_4 (0·022 M, pH2·1) will rapidly oxidize α-glycols, α-hydroxyaldehydes and α-hydroxyketones and diketones, quoting Fleury and Courtois (1950) as his authority. According to these authors (Fleury *et al.*, 1949) α-aminoalcohols, α-aminoaldehydes and α-aminoketones require a neutral or slightly alkaline medium (pH 7-8). According to Dyer (1956), however, α-glycols and α-aminoalcohols will be nearly completely oxidized in 5-10 minutes if excess HIO_4 is present, as it is when the reaction is performed histochemically. Longer than 10 minutes is required for the oxidation of α-hydroxyaldehydes, α-hydroxyketones, α-ketoaldehydes and diketones and their amino derivatives.

This information appears relevant in the light of results obtained by Leblond and his colleagues, which have already been considered in this chapter.

Temperature. If used at high temperatures for long periods, periodic acid probably oxidizes aldehydes to carboxylic acids, and perhaps even oxidizes groups other than the specific 1 : 2-glycol group in a similar manner. If this is true the effect shown by Dempsey *et al.* is likely to be due to carboxylic acids as well as to the oxidation products of sulphydryls and disulphides. Whatever the true explanation of the observations made by these authors, their importance is undeniable. They indicate that in the histochemistry of polysaccharide-containing substances periodic acid solutions must be used for short periods only.

The temperature of the reaction needs only brief consideration. An increase of only a few degrees rapidly increases the rate of oxidation, and probably affects its specificity for the conversion of 1 : 2-glycol groups to dialdehydes in the two ways suggested above. **In the histochemical use of the PAS reaction for substances in Groups I, II, and III, therefore, nothing higher than room temperature should be employed and the time of oxidation should be restricted to 5 minutes or less.** When substances in Groups IV and V are being investigated, however, the time of oxidation must be increased if false negative reactions are to be avoided.

Reducers. The use of a reducing rinse was first suggested by

Hotchkiss (1948) for the purpose of removing periodate or iodate remaining combined in the tissues after the periodic acid bath, since iodates and periodates restore the colour to Schiff's solution. Hotchkiss' rinse contains KI and $Na_2S_2O_3$, in 60 per cent. alcohol with 0·02 N HCl. and it acts as an iodide-thiosulphate solution in which the maximal amount of acid compatible with the thiosulphate is incorporated. It reduces both periodates and iodates when in the acid state and must be reacidified when its acidity falls. An objection to its use, suggested by McManus (1948b), is that it decreases the amount of aldehyde available for colouring the Schiff reagent and thus decreases the brilliancy of the stain. Hotchkiss himself suggested that prolonged rinsing in 70 per cent. alcohol or in water might be adequate to remove trapped periodate and iodate. McManus preferred to avoid either type of rinse since he could show that control sections placed in 0·5 per cent. sodium periodate or potassium iodate for 5 minutes gave a negative Schiff reaction. There is no doubt that the aldehydes formed by the periodic acid oxidation are slowly destroyed by Hotchkiss' reducing rinse. Though formerly I considered the reducing rinse essential for the histochemical, as opposed to the histological, use of the PAS reaction, I no longer hold this view. If a reducing rinse is used it is reasonable to follow McManus (1954) and to designate this by the inclusion of the letter R to make the complete reaction PARS, but for most purposes the standard PAS method (Appendix 9, p. 832) should replace it.

Schiff's Reagent. The preparation of Schiff's solution has been considered in Chapter VIII and methods are given in Appendix 8. It may be stressed once more that various batches of the reagent vary considerably in performance. Some workers prefer to dilute the original solution with an equal part of distilled water, slowing its action and allowing greater flexibility. I prefer to test each new batch with a known control section, containing a large amount of the substance for which the PAS reaction is to be employed, and to calculate the optimum time of immersion from this. For human mucoprotein materials an average time of 5–6 minutes is usual with the de Tomasi variant of Schiff's solution.

Sulphite Baths. In the Feulgen reaction the Schiff bath is followed by two or three washes in sulphite water before dehydration and mounting are carried out. Both Hotchkiss and McManus employed these baths in their techniques for the PAS reaction and Lillie (1947, 1948) also employed them in his version. McManus (1948a) noticed that washing in running water for 5–10 minutes after the last sulphite bath enhanced the final colour considerably. For routine use in histology or histochemistry, I consider that the sulphite baths are unnecessary and that their place may be taken by the 10-minute wash in running water.

Interferences. The interference produced by periodate and iodate has already been considered above. That produced by the presence of acetal phosphatides (plasmalogens) has been mentioned in Chapter VIII, and these substances are fully considered in Chapter XII. This type of interference is considerably greater in frozen than in paraffin sections. If unoxidized sections, treated with Schiff's reagent, indicate the presence of a considerable amount of plasmal aldehyde, a number of expedients may be employed for its blocking or removal. These are considered in Chapter XII, in the section on blocking techniques for aldehydes.

Methods for Increasing the Specificity of the Reaction. It was suggested by Gersh (1949) and by McManus and Cason (1950) that confirmation of the fact that a given PAS reaction was due to the presence of 1 : 2-glycol groups might be obtained by acetylation of these groups and the consequent blocking of their oxidation by periodic acid. Gersh himself used 2 per cent., 10 per cent., or 99 per cent. acetic acid, or acetic anhydride, for this purpose, remarking that although it was unusual for simple carbohydrates to form compounds with acetic acid, the negative PAS reaction after this treatment was understandable only on such a basis. He observed that if the acetylated sections were subsequently treated with 0·1 N HCl the 1 : 2-glycol group was restored and reaction with periodic acid could once more take place. I employed 10 per cent. acetic anhydride in dry pyridine for 1 hour at room temperature to effect acetylation of the 1 : 2-glycol groups responsible for the PAS reaction (Pearse, 1950b) but, as pointed out by Lillie (1954), the strength of the acetic anhydride and the time of incubation were inadequate for complete acetylation of hydroxyl groups though probably adequate for NH_2 groups. Lillie uses 40 per cent. acetic anhydride in anhydrous pyridine for 1–24 hours at 25° or for ½ to 6 hours at 58°, and this formula is recommended. McManus and Cason (1950) used dilute alkali (0·1 N KOH) to reverse the acetylation process and concluded that any material giving a colour with PAS, obliterated by acetylation and restored by subsequent treatment with alkali, possessed numerous 1 : 2-glycol groups and was likely to be carbohydrate. They supposed that acetylated amino and substituted amino groups would be less easily deacetylated than the hydroxyl groups of unsubstituted 1 : 2-glycols and that acetylation followed by deacetylation, HIO_4, and Schiff's reagent would distinguish 1 : 2-glycols from α-amino alcohols. As pointed out by Hale (1957) this is not true, since periodic acid will react as soon as the adjacent OH is deacetylated. Weak NaOH or KOH have usually been employed for deacetylation. Lillie (1954) recommends treatment with an alcoholic ammonia for the same purpose (see Appendix 9, p. 832). Hale (1953) considered that acetylation blocked all potentially PAS-positive substances and that

deacetylation restored their activity. I considered and still consider, with Lison (1953), that the whole process is of academic interest rather than practical utility.

Fisher and Lillie (1954) found that prolonged methylation (48 hours) prior to oxidation (see Chapter V, p. 123 and Appendix 5, p. 801) would abolish the PAS reactivity of most tissues. Lillie (1954b) noted that 24 hours' methylation after HIO_4 oxidation would also block the reaction. This latter effect is due to the formation of acetals:

$$RCHO + 2\ CH_3OH \longrightarrow RCH(OCH_3)_2 + H_2O$$

which occurs readily on warming aldehydes with alcohols in the presence of mineral acids. The mechanism of premethylation blocking has not been explained.

Objections to the Specificity of the Reaction. Jeanloz (1950) raised objections to the use of the PAS reaction for identification of polysaccharide structure on the grounds that the presence of two adjacent free OH groups (1 : 2-glycol) in the carbon chain bore no relationship to the positive reaction. He stated that numerous sugars possessing such groups, cellobiose and methyl α-glucopyranoside for instance, gave a negative result, while the consumption of HIO_4 by substances which react positively varied from 1 molecule per hexose residue in the case of starch, glycogen and cellulose to 0·1 to 0·4 molecules per hexose residue in the case of hyaluronic acid and chitin. McManus and Hoch-Ligeti (1952), however, using a modification of the Hotchkiss spot test, obtained a positive PAS reaction with cellobiose, and hyaluronic acid and chitin have now been shown to be PAS-negative. It is clear that consumption of HIO_4 need not be related in any way to the PAS reaction since the latter will be negative however much or little HIO_4 is involved unless the reaction product is a stable non-diffusible aldehyde.

Compounds containing pentose sugars with unsubstituted glycol groupings may give a positive result in fixed tissues. The methylpentose sugar fucose has already been shown to do this (Leblond *et al.*, 1957). Compounds such as adenosine-5-phosphoric acid (muscle adenylic acid) and adenosine triphosphate, in which the 1 : 2-glycol of D(-) ribose is free, give a positive result with the PAS reaction *in vitro*. In frozen sections especially, the A bands of voluntary muscle are strongly stained with PAS even when the glycogen they contain has been removed. Since hexose-containing glycoproteins are not known to be present in this situation, despite the assurances of Hotchkiss that substances of low molecular weight are removed during fixation, dehydration and embedding, it is possible that the reacting substance is protein-bound adenylic acid. Bearing these possible exceptions and lipids of various classes in mind, a positive PAS reaction must be

considered to be due to the presence of hexoses. The part played by hexosamines appears to be unimportant and hexuronic acids can be regarded as non-contributory.

It must be emphasized that differences in the PAS reactions of given structures, reported by different workers, are as much due to variation in technique as to variation in the structures themselves. Paneth cell granules and blood and tissue eosinophil granules are often reported as staining positively. The former undoubtedly vary depending on the species from which they are derived and on the type of fixative employed (Lillie, 1950). In human intestine they can be made positive or negative by varying the solvent used for the periodic acid and the time of oxidation. On the other hand, the reported differences in the case of human Russell bodies are equally due to variation in these structures themselves since they may be composed of strongly or weakly staining mucoprotein or glycoprotein, or of PAS-negative simple protein. Only a proportion of mast cells, demonstrable as such by their metachromasia, stain by the PAS method, and in many of these the colour developed is weak. Gomori (1952) considered that this was due to the fact that fully substituted (sulphated) heparin is PAS-negative (Jorpes *et al.*, 1948). Heparin monosulphate, however, was observed by these last authors to be PAS-positive *in vitro*.

Effect of Formaldehyde on the PAS Reaction. Hale (1953) observed that an increase in intensity of PAS staining followed pretreatment of sections with 0·2 N-NaOH for 15 minutes at 22°. He suggested that by this means three types of mucin could be distinguished. The first, which showed a marked increase in colour, consisted of long chain mucopolysaccharides such as those presumed to occur in human rectal mucin. The second, in which the increase was small and inconstant, he regarded as mucoproteins and the third, which showed no change, was thought to include the glycoproteins. In a later paper (1955) Hale showed that the effect of alkaline hydrolysis in increasing the colour developed in the PAS reaction could be attributed to the reversal by this procedure of blocking induced by formalin fixation. The possibility was considered that this effect of formaldehyde might be due to the production of acetal or hemiacetal groups from the vicinal OH groups of the glycols or to polymerization. The results of experimental procedures suggested that the second hypothesis was the correct one.

Very little work on these lines appears to have been carried out by other authors but Holczinger (1956) reported an *increased* staining of reticulin fibres in man and the rat after fixation with formalin. These contradictory opinions do not appear to have been resolved.

For non-sulphated epithelial mucins or mucoproteins the PAS reaction has no peer (Plate IB) ; its sensitivity is such that single

mucous-secreting carcinoma cells can be detected far more easily than with any other method, especially in exudates into the serous cavities. For the acid mucopolysaccharides of the connective tissue mucins, on the other hand, the PAS reaction is useless. The wide application of the reaction in histochemistry is illustrated by the use of the method in preparing the materials illustrated in Plates Ia, Ib and Id and Fig. 208.

Alternative Oxidizing Agents. Lead tetraacetate ($PbAc_4$) was first used by Criegee (1931, 1948) for chemical estimations on 1 : 2-glycols. It has also been extensively used by Perlin (1955) for elucidation of the structure of reducing disaccharides. Each position of the glycosidic linkage is associated with a characteristic oxidation pattern. A number of authors have applied the reagent to the histochemical study of carbohydrates (Crippa, 1951 ; Glegg *et al.*, 1952 ; Shimizu and Kumamoto, 1952 ; Jordan and McManus, 1952 ; Lhotka, 1952d ; Hashim and Acra, 1953 ; Graumann, 1953a and b). Originally the reagent was used as a 1 per cent. solution in acetic acid but later workers added sodium or potassium acetate following the finding by Criegee and Buchner (1940) that cleavage of 1 : 2-glycols was greatly accelerated by the presence of these compounds. Hashim and Acra (1953) found that strong solutions of acetic acid caused swelling of the tissues and slowed the rate of the reaction. They therefore diluted the solution with water or organic solvents (benzene, toluene). Casselman (1954) made a comparative study of the various methods employing $PbAc_4$. He found that starch and glycogen did not give consistently positive or negative results. Their reaction depended on the conditions of oxidation. The least active, but most specific reagent, leaving starch and glycogen unstained, was the simple solution in acetic acid (glacial). If acetate was added both substances tended to give a weak positive reaction. Dilution with water increased the rate of oxidation and produced a regularly positive reaction with starch and glucogen.

It was noted by Criegee (1935) that, in addition to 1 : 2-glycols, $PbAc_4$ would readily oxidize α-hydroxy acids (CHOH.COOH) whereas HIO_4 would more readily oxidize α-hydroxyaldehydes and ketones (CHOH.CHO,CHOH.CO). Lhotka (1957) therefore, suggested that the difference between PAS and PA,$PbAc_4$,S should indicate the amount and location of α-hydroxy acids. He was unable to observe any difference, however, in practice. In a previous paper (1952c), following the observations of Hockett and McClenahan (1939), Bell and Baldwin (1941) and Hockett *et al.* (1943), Lhotka had shown that with $PbAc_4$ in acetic acid the respective oxidation times for *cis* and *trans*-glycols were 15 and 30 minutes (see formula for β-D-glucose on p. 239 ; this is written as a *trans*-glycol with the hydroxyls in the 2- and 3-positions on opposite sides of the ring). He suggested that distinction between the

two might be made by these means but little use seems to have been made of this principle. Owing to overlap in the middle of the range it would be necessary to confine oxidation to less than 5 minutes to be reasonably certain that mainly *cis*-glycols were reacting. This period of oxidation is quite inadequate ; Glegg *et al.* (1952) used 1-4 hours and Crippa (1951) 30 minutes.

The reactions of boric acid with polysaccharides were recorded by Deuel *et al.* (1948). They noted that cyclic glycols with the *cis*-configuration formed complexes with boric acid much more easily than did *trans*-glycols. One boric acid molecule combines with one or two glycol molecules. Staple (1955, 1957) was unable to use this reaction to block the oxidation of glycols with $PbAc_4$ but he found that treatment with 0·1 to 0·001M-boric acid *during* or *after* oxidation would prevent subsequent reaction with Schiff's reagent only in certain structures. The effect was better demonstrated with alcoholic than with aqueous Schiff's reagent. It seems that Staple's results are similar to those obtained by the interposition of various aldehyde-blocking reagents after HIO_4 oxidation and that, as with these, interpretation of the effect is complicated by steric factors which are difficult to assess.

Other oxidizing agents which have been employed histochemically for cleavage of 1 : 2-glycols include sodium bismuthate (Lhotka, (1952e) manganese acetates (Lhotka, 1953) and phenyl iodosoacetate (Lhotka, 1954). The first of these, which was used by Rigby (1949, 1950) in chemical studies, apparently acts by forming a cyclic compound with the glycol which then splits to form 2 carbonyl compounds and bismuth pentoxide. The reaction takes place in aqueous or organic media but Lhotka found that the best medium was 20 per cent. orthophosphoric acid. His results were essentially similar to those produced by HIO_4 and $PbAc_4$. Zonis and Pesina (1950) and Zonis and Kornilova (1950) showed that the tri- and tetraacetates of manganese would split 1 : 2-glycols to produce aldehydes and Lhotka (1953) applied this reaction to the histochemical demonstration of glycols. He stated that these reagents could not replace HIO_4 and $PbAc_4$ but that they might prove useful as supplementary reagents if their specificity became better understood. In further studies devoted to the testing of alternative oxidants Lhotka (1954) used phenyl iodosoacetate prepared by the method of Pausacker (1953). He found that a 0·02M solution in acetic acid produced the optimal degree of oxidation in the minimum time. Owing to the fact that it is necessary to synthesize both this and the manganic acetate reagents they have not been further tested and no valid opinion as to their value in carbohydrate chemistry can be given at the present time. A number of alternative oxidants used in polysaccharide chemistry have not been tested in histochemical practice.

Among these are the Midas reaction (H_2O_2 and OsO_4 in *tert*-butanol) and perbenzoic acid in $CHCl_3$ at 0°.

Metachromasia

Histochemically metachromasia can be defined as the staining of a tissue component so that the absorption spectrum of the resulting tissue-dye complex differs sufficiently from that of the original dye, and from its ordinary tissue complexes, to give a marked contrast in colour. As such, it may be produced by a wide variety of dyes (Lison (1935b) gives a full list), but for practical purposes " metachromasia " is conveniently limited to the effects seen with dyes of the thiazine group only, and here it refers to a change of colour from blue, through violet, to red, occurring when tissues are stained in dilute aqueous solutions of dyes of this group.

TABLE 16

Absorption Spectra of Metachromatic Dyes (*After Kelly*, 1956)

Dye	Absorption Maxima (mμ)	
	Orthochromatic	Metachromatic
Toluidine blue . .	630	480–540
Azure A . . .	620	480–530
Methylene blue . .	665	570
Cresyl blue . . .	625	530
Crystal violet . .	590	510
Basic fuchsin . .	543	510
Thionine . . .	597	557

With the thiazines the mechanism underlying the production of metachromasia is better understood than with the unrelated triphenylmethane dyes (methyl violet) or azines (neutral red). The metachromasia of the triphenylmethanes is important histochemically only in the case of amyloid, and the reactions of this substance are dealt with in Chapter X. On account of its practical simplicity thiazine dye metachromasia has been extensively employed as a histochemical method and there seems to be no falling off in the number of applications reported in the literature. A much greater volume, of paper at least, has been covered by articles and reviews dealing with the mechanism of the reaction and it may be well to consider the latter at this point. Reference should be made, by those especially interested in metachromasia, to the origin papers of Sylvén (1941, 1945, 1954), Kramer and Windrum (1955), Kelly (1956), and Schubert and Hamerman (1956).

Mechanism of the Metachromatic Reaction. Michaelis (1902) suggested that metachromasia with the thiazine dyes was due to the formation of tautomers of the dyes employed which possess absorption spectra differing from the original and Holmes (1928) showed that this explanation was probably true in the case of the oxazine dye brilliant cresyl blue. More modern work by Michaelis, in collaboration with Granick (1945), has introduced the conception of polymer formation. The monomeric form of the dye is blue or violet, the dimers and trimers are progressively more violet and the polymers red or pink. Polymerization of the substrate, with which the dye combines, induces polymerization of the dye, and hence metachromasia. Contributions to this theory have been made by Bank and Bungenberg de Jong (1939), Rabinowitch and Epstein (1941), Sheppard and Geddes (1944a and b), Vickerstaff and Lemin (1946), Massart *et al.* (1951) and Sylvén and Malmgren (1952).

Following Michaelis (1947) it is possible to recognize two varieties of metachromasia in histochemistry. This author showed that toluidine blue has an absorption spectrum with three bands, alpha, beta and gamma. The monomeric alpha form is blue, and dimeric beta form violet and the polymeric gamma form is red. The production of γ-(red) metachromasia with toluidine blue in tissue sections is due to predominance, in or on the substance stained metachromatically, of the γ form of the dye. The purple β-metachromasia is not due to predominance of the β band since, as Schubert and Hamerman have pointed out, this actually disappears with formation of the γ band. It is presumably due to the simultaneous presence of the γ- and α-forms of the dye. Provided that this is recognized there is no harm in using the terms γ- and β-metachromasia to refer to the red and purple types respectively, as far as tissue sections are concerned.

Sylvén (1954, 1959) regards metachromasia as "a special type of orderly dye aggregation characterized by the formation of new intermolecular bonds between adjacent dye molecules." The molecules of the substrate he regards as centres of orientation whose free anionic groups will attract the polar groups of the dyestuff, as postulated by Sheppard (1942) and as indicated in Fig. 76, below, left. This is the primary requirement for metachromasia.

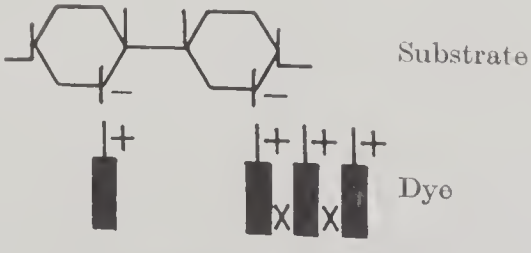


FIG. 76. X = Water molecule.

Aggregation of the dyestuff, the secondary requirement for metachromasia, is shown in the right-hand part of the diagram. It is favoured by high dye concentrations, and by high pH levels which favour the maintenance of a higher degree of hydration. Sylvén stresses the fact that the presence of water is essential for metachromatic interaction.

For the first stage of metachromasia to take place a certain minimum surface density of negative charges on the substrate is necessary. In hyaluronic acid, where the distance between COOH groups is about 10·3Å, no metachromasia occurs in dilute aqueous solutions. In the case of pectic acid, a straight chain uronic acid polymer, the intercharge distance between COOH groups is about 5Å and this is enough to produce a weak metachromasia. Polysaccharides containing alternate SO_3H and COOH groups would be expected to have an intercharge distance of about 4 and 6Å and to show a moderate degree of stable metachromasia. With the introduction of two or three SO_3H groups per disaccharide unit the intercharge distance is reduced to less than 4Å and this results in a strong and stable metachromasia.

Sylvén considers that the spectral shifts which constitute metachromasia are due to the formation of new bonds, with higher bonding energy, between the aggregated dye molecules. Because of their low energy and reversibility these are considered to be secondary valency bonds (van der Waal's forces). For their occurrence certain minimal energy requirements must be satisfied. Complete metachromasia over a substrate, without admixture of orthochromatic components, is first reached when the γ-band prevails. This occurs at about 520 mμ which corresponds to about 8 cal/mol, according to calculations based on Planck's formula. Table 17, below gives some of Sylvén's calculations which serve to illustrate this important conception.

TABLE 17

Energy requirements for production of metachromasia

Absorption maxima (mμ)	Δ E (cal./mol.) E max — E 615 mμ	Maximum absorption of purified compounds
600	1·0	—
580	2·8	RNA
560	4·5	Pectinate
540	6·4	Chondroitin sulphate
520	8·5	KSCN, detergents
500	10·6	Heparin
480	13·0	Trisulphonated HA

The conception of polymerization of the dye, which is inherent in the above-mentioned work, is strongly held by the majority of workers

in the field. The question of polymerization of the substrate is another matter. Sylvén and Malmgren (1952) found that metachromasia was more easily produced when their substrate (S-free hyaluronic acid) was present in gel form. Concentrated gels and dried films showed marked γ metachromasia though solutions were devoid of this effect. It was observed that the introduction of more COOH, PO_4 or SO_3H groups produced metachromasia even in the sol state, and this agrees with the observations of Walton and Ricketts (1954) who tested the precipitation of toluidine blue *in vitro* by dextran polymers and their sulphated and carboxylated analogues. They found that the amount of dye precipitated was a linear function of the concentration of the polymer and its SO_3H content. The degree of polymerization had no effect on the metachromasia of the precipitates and Walton and Ricketts concluded that if, under histochemical conditions a tissue component showed a fall in its metachromatic reaction this could mean (1) Decrease in concentration of acidic polysaccharide or (2) Loss of acidic groups by protein binding (see below), or (3) Disruption of the molecular structure of the polysaccharide or degradation to diffusible fractions. It did *not* mean depolymerization of the polysaccharide. Walton and Ricketts also found that one molecule of the dye reacted with one acidic group of the substrate which agrees with the later conception of Michaelis (1950) that dye is distributed in monomolecular fashion over the surface of the substrate. Schubert and Hamerman (1956) pointed out that Walton and Ricketts in their work on dextrans were measuring the amount of dye precipitated and not metachromatic activity and that the two were not necessarily the same. They also point out, and this should always be remembered, that there is no method known by which the degree of metachromasia can be measured since the dyes concerned do not obey Beer's law in aqueous solutions.

To sum up ; from the theoretical point of view metachromasia signifies *only* the presence of free electronegative surface charges of a certain minimum density. The older theory of specific groups cannot be sustained. Negative metachromasia *may* mean bound or masked electronegative surface charges, or the absence of these from the material being examined.

The Practice of the Metachromatic Reaction. *Fixation.* This subject has already been discussed in Chapter IV, p. 70, where some selective fixatives for acid mucopolysaccharides have been listed. Many studies on metachromasia are still carried out on paraffin sections of tissues fixed in the standard medium used in the department concerned and it is not possible to be dogmatic in this matter. It must be emphasized, despite the opposite views of a number of authorities, that fixation is still of considerable consequence and that lesser degrees of meta-

chromasia can easily be abolished by fixation and subsequent embedding. I would like to enter a plea for the extended use of fresh, thin, cold microtome sections in studies on metachromasia. These should be used in pairs, one section totally unfixed and the other rapidly fixed in one of the preferential fluids such as Lillie's alcoholic lead nitrate (p. 789). In this way a check can be made on water-soluble substances which may be lost even during the brief staining involved.

Concentration of the Dye. The ratio of dye to substrate, or chromotrope as it is often called, has considerable influence on the production of metachromasia (Levine and Schubert, 1952 ; Schubert and Levine, 1953, 1955 ; Michaelis, 1950) and if insufficient dye is present metachromasia will not occur (Sylvén and Malmgren, 1952). The usual concentration of dye is of the order of 0·1 per cent. but even 0·01 per cent. is adequate (Kramer and Windrum, 1955). Following the example of Sylvén (1941, 1945) the solvent now commonly used is 30 per cent. alcohol. Chromatographic and spectrophotometric investigations carried out by Ball and Jackson (1953) have drawn attention to the unsuitability of some samples of toluidine blue for use as metachromatic stains. Most samples of toluidine blue perform adequately and contain, besides the metachromatic component, only non-staining fractions. Other dyes, such as thionin, contain different staining fractions which have different colours. A metachromatic effect with such dyes may be due to differential staining.

Duration of Staining. For the dye concentrations usually employed a time of 5-20 minutes is appropriate but with very dilute solutions (0·001 per cent.) times of up to 4 days have been used. Kramer and Windrum point out that overstaining of strongly metachromatic tissue components leads to an appearance of orthochromasia. Thus the staining time should not only be related to the concentration of dye but also to the type of tissue component being studied.

Post-Treatment of Sections. In the first edition of this book it was stated, in strong terms, that " alcoholic dehydration was capable of removing all metachromasia " and that the process of differentiation thus involved should be avoided by examination of the sections in water, after staining. Exception has been taken to this statement, or rather to the manner in which it was stated, and so it is necessary to say it again in different terms. The conception of Sylvén (1941, 1945) that alcohol-resistant metachromasia is " true " and alcohol-labile metachromasia is " false " must be regarded as a definition, which can be accepted or rejected. Kramer and Windrum (1955) support this definition and have performed experiments to prove it. They contend that alcohol *never* reverses metachromasia due to ester sulphates. Others (Baker, 1951 ; Hess and Hollander, 1947 ;

Landsmeer, 1951 ; and Wislocki and Singer, 1950) consider that alcohol may abolish metachromasia and, on the other hand, Persson (1953) has noted that the metachromasia of sulphate-free hyaluronate is not abolished by alcohol.

My own objections were and are merely to the use of the words true and false in describing the effects of alcoholic dehydration. I did not and do not disagree with Sylvén's contention that alcohol-fast metachromasia is most likely to be due to the presence of sulphate esters. With Kelly (1956) I still feel that it is essential to examine sections first in water, or in the solvent in which they were stained. After noting, and if necessary recording photographically, the metachromasia present, the section can be mounted in a medium such as Apathy's syrup or dehydrated and mounted in a synthetic resin. According to Kramer and Windrum these two procedures have similar effects. In animal tissues strong γ-metachromasia in water-mounted sections may be due to ester sulphates or to materials containing other acidic groups. β-metachromasia in such tissues is due to the presence of the weaker acidic groups (PO_4 ; COOH) in high local concentration. Alcohol-fast or Apathy-stable γ-metachromasia is largely due to ester sulphates but it has not yet been shown that some of the metachromasia of these is not extinguished by differentiation. Alcohol-stable β-metachromasia is normally exhibited only by nucleoproteins as first observed by Wislocki, Bunting and Dempsey (1947).

Ionic Competition. Lison (1953), in the second edition of his book, agrees that his original idea that stable metachromasia was the sole property of higher esters of sulphuric acid cannot be maintained. He points out, however, that where the metachromasia of polysaccharides is due to carboxyl groups it is easily reversed by treatment with 1 per cent. NaCl and suggests that this may form the basis of a differential method. Sylvén (1954) also comments on the ease with which small concentrations of salt will abolish or *prevent* a metachromatic reaction. I would prefer to see a specific method for ester sulphate evolved by using this last principle, staining in the presence of low concentrations of NaCl just sufficient to prevent metachromasia due to other groups from occurring. However, Sylvén also points out that different ester sulphates exhibit a wide degree of difference in stability to salts (and also to alcohol). This might make determination of the precise salt concentration a matter of some difficulty.

Protein Competition. It has been conclusively demonstrated by Kelly (1955), Hamerman and Schubert (1953) and French and Benditt (1953) that the addition of soluble proteins will prevent metachromasia. Competition between the dye and the protein for the available acid groups of acidic polysaccharides is markedly modified by pH. In the case of histone elevation of the pH favours the dye and lowering of the

pH favours the protein, but Noguchi (1956) has shown that anionic polysaccharides combine with proteins even on the alkaline side of the I.E.P., where both have negative net charge. This principle has not been applied to the problem of increasing the specificity of the metachromatic reaction.

Effect of Methylation. The metachromasia of the mucins of stomach, intestine, mixed tumour stroma and cartilage was found by Fisher and Lillie (1954) to be decreased by 1 hour's methylation at 58° (Appendix 5, p. 801) and abolished by 2 hours' treatment. That this effect is not due to desulphation is indicated by the fact that demethylation restores the metachromasia in its original intensity. It is thus likely to be due to methylation of the SO_3H groups of the polysaccharides.

Sulphation-induced Metachromasia

Metachromasia in polysaccharides after sulphation was first shown by Bignardi (1940a and b). Following the demonstration by McManus and Mowry (1952) that treatment of tissue sections with sulphuric acid would induce hæmatoxylin-staining of basement membranes Kramer and Windrum (1953, 1954) developed the sulphation-metachromasia method as a histochemical technique. Several methods of sulphation were employed :—(1) Conc.H_2SO_4, followed by washing in water ; (2) Chlorosulphonic acid in pyridine (Ricketts, 1952) ; (3) Sulphuric acid-acetic anhydride (Schrauth, 1932) ; (4) Pyridine-sulphur trioxide (Baumgarten, 1926) ; (5) Chlorosulphonic-acetic-chloroform; (6) Sulphuric acid-acetic anhydride-ether. Of these the best for routine use was found to be the last. This closely resembles Gomori's sulphuric-ether mixture which can be substituted for it.

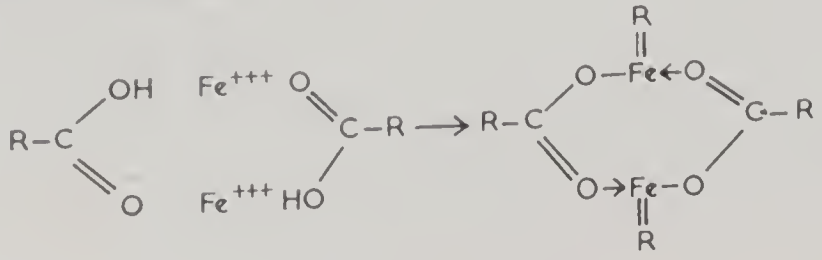
Staining after sulphation was carried out with 0·01 per cent. Azure A in 30 per cent. alcohol and an intense γ-metachromasia was induced in basement membrane, reticulin, gastric and Brunner's gland mucin, and in thyroid and pituitary colloid *inter alia*. Collagen remained orthochromatic. The parallel between PAS staining and sulphation-metachromasia is very close and the methods can be used in parallel but the possibility that other than polysaccharide OH groups may be sulphated by the above techniques and contribute to the observed metachromasia has not been considered. Reitz *et al.* (1946) were able to sulphate the OH groups of serine, threonine, hydroxyproline and hydroxylysine, using conc. H_2SO_4 at low temperatures. The negative metachromasia of sulphated collagen, which contains a large amount of hydroxyproline and serine, would appear to dispose of the idea that protein-OH groups play much part in sulphation-metachromasia. The techniques of sulphation are given in Appendix 9, p. 835.

Methylene Blue Extinction (MBE)

Further assistance in the identification of substances in Group II can be obtained by a study of their dye-binding capacity for methylene blue measured at various pH levels. It has long been customary to use the basophilia of the acid mucopolysaccharides as the basis of a qualitative procedure (e.g. the hæmatein and carmine of the older mucin stains, which depend upon it). More recently, using the method originated by Pischinger (1926, 1927), Dempsey and Singer (1946), Dempsey *et al.* (1947), and Dempsey, Singer and Wislocki (1950) have used a technique for making quantitative estimation of the degree of basophilia registered by tissue compounds. They stained sections with M/2000 methylene blue at various pH levels for 24 hours, mounting them with minimum dehydration, and measured (usually spectrophotometrically) the amount of dye bound by various tissue components. I have employed this method, without spectrophotometry, using as end point the virtual extinction of binding capacity for methylene blue (Pearse, 1949a, 1950a). The MBE method makes possible a comparative estimation of the degree of basophilia manifested by various structures and it is particularly useful for distinguishing between acid mucopolysaccharides and mucoproteins. Dempsey and Singer (1946) found that mast-cell granules and the ground substance of cartilage were able to bind methylene blue at pH 2·0, whereas the binding capacity of tracheal mucus was extinguished at pH 3·0 and that of thyroid colloid ribonucleic acid at pH 4. Using the same solutions as the above authors I have found, however, that the RNA of plasma cells still binds methylene blue at pH 2·6. Friedenwald (1947), who has shown that the distinction between metachromatic and orthochromatic staining of tissues can be exaggerated by staining in the presence of high concentrations of magnesium, has also shown that at pH 1·5 nucleic acids fail to stain if the solution is saturated with magnesium chloride, whereas cartilage and mast-cell granules still stain. He suggested that this was due to blockage of the phosphoryl groups of the nucleic acids by magnesium. Using a method such as this, distinction can be made more easily between the sulphuric acid esters and other basophilic and possibly metachromatic substances. **If nucleic acids can be excluded the capacity to bind methylene blue below pH4 almost certainly indicates sulphate groups and thus acid mucopolysaccharides.** An exception to this is the dye-binding capacity of certain substances, presumed to be lipid complexes, which are occasionally found in tissue sections. The lipochondria of Ries (1935) and Holftreter (1947) belong to this group.

Hale's Method for Acid Mucopolysaccharides

The dialysed iron method, introduced by Hale (1946), depends on the affinity of free acidic groups in the tissues for colloidal Fe^{+++} at pH2. After washing, bound ferric iron is demonstrated by means of the Prussian Blue reaction. This gives a particularly easily visible and generally clean result. Originally Hale maintained that this method would demonstrate non-sulphated hyaluronic acid as well as sulphated mucopolysaccharides in Carnoy-fixed tissues. The specificity of the method seemed doubtful and Lillie and Mowry (1949) investigating the absorption of iron by tissue sections using M/100 ferric chloride in 1 per cent. mannitol (pH5) found that mucin, collagen and reticulin were selectively impregnated. In similar experiments (1951) I could not find that the affinity of the tissues for iron was limited to the acid mucopolysaccharides. Because of this I noted in the first edition of this book that the usefulness of the method was restricted. Lison (1953) observed that nucleoproteins and other proteins were stained by the method and Lillie (1954) agreed that the method was not selective for acid polysaccharides. Mancini (1950), however, and Gomori (1952) found that staining was largely confined to those structures which gave metachromasia with toluidine blue. Davies (1952), using various preparations of hyaluronic acid on slides, found that all gave an intense reaction. He found also that a positive reaction was given by fibrin, gelatin, casein and even by peptone. Braden (1955) confirmed these results and disagreed with Immers (1954) whose experiments had upheld the specificity of the reaction. The specificity of iron staining in general has been discussed at length by Wigglesworth (1952). Following Fischer and Hultzsch (1938), this author suggests that there is no doubt that iron will be bound by any monoesters or diesters of phosphoric acid present in fixed tissues and available for reaction. In the case of binding to protein (amino-acid) groups, however, there is considerable lack of agreement. Bechtold (1928) found that constant amounts of iron were firmly held by proteins and he considered that this was due to electrostatic absorption to the amino and carboxyl groups of the protein. Other authors consider that chemical combination between protein and iron takes place. Wigglesworth believes that with ferric iron the initial linkage is an electrostatic bond with the free carboxyl of the protein. Subsequently chelate complexes of the type shown below are formed.



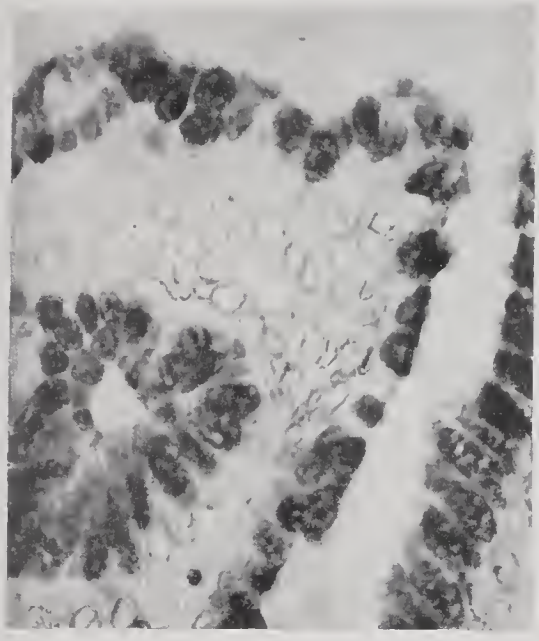


FIG. 74. Human stomach mucosa (5·5 μ paraffin section). Subjected to 16 hours' benzoylation at 22°. Intracellular mucin granules are strongly stained. Coupled tetrazonium reaction. × 400.

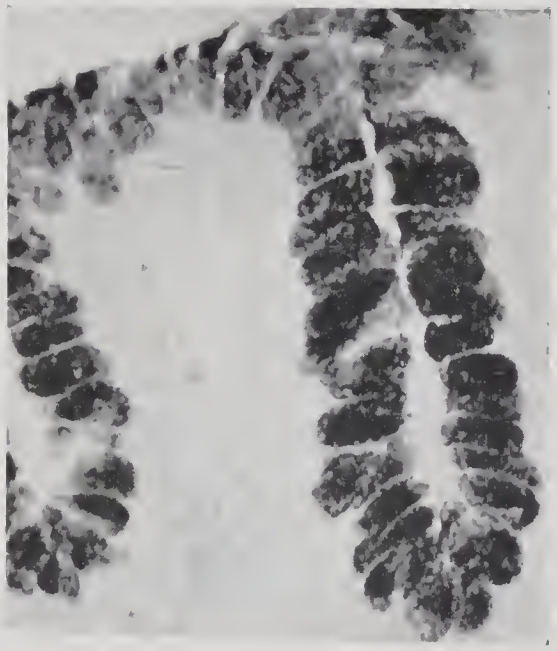


FIG. 75. Similar section to Fig. 74. Showing similar staining of intracellular mucin. Eosin-Gram-Weigert method. × 400.

[*To face p.* 256.

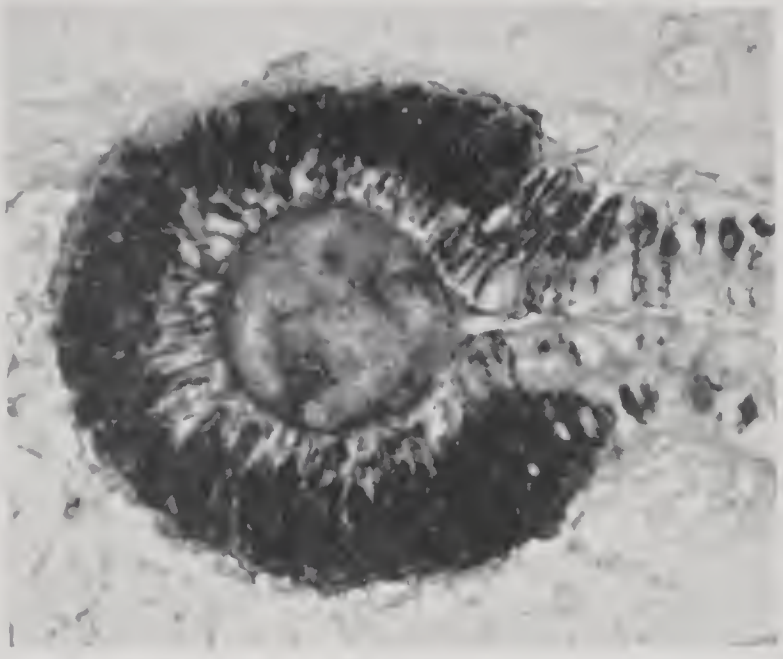


FIG. 77. Snail (*Helix pomatia*). Diverticulum of intestine. Four μ fresh frozen section. Shows intense staining of intracellular mucins. Dialysed Iron method. × 130.

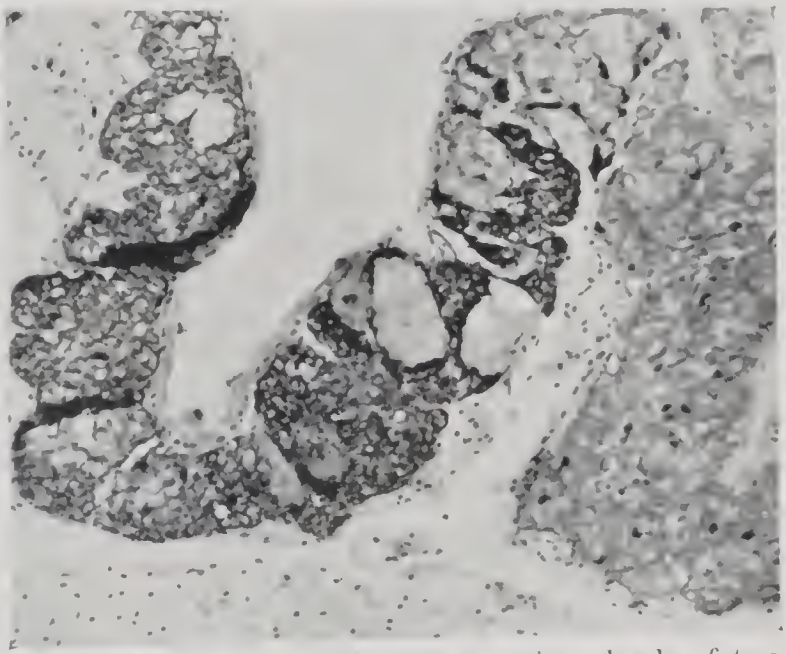


FIG. 78. Snail (*Helix aspersa*). Mucous secreting glands of two types are shown. Alcian green—haemalum. × 126.

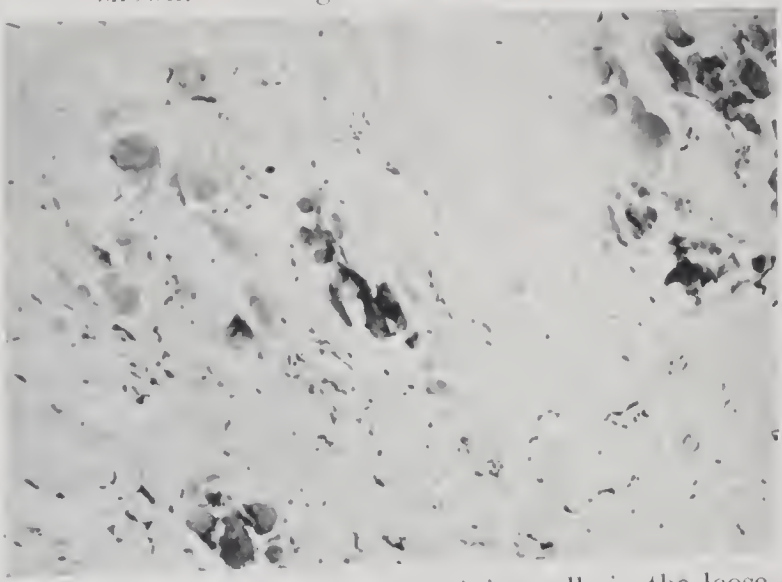


FIG. 79. Snail (*Helix aspersa*). Mucin-staining cells in the loose connective tissues of the foot. Germanate method. × 130.

Wigglesworth emphasizes the fact that the formation of complexes of this kind must depend ultimately on steric factors and these have not been critically considered as yet. According to Sidgwick (1950) the affinity of Fe^{+++} for COOH is so great that calcium oxalate will dissolve in solutions of $FeCl_3$. *Per contra* oxalic acid will remove protein-bound iron from hæmosiderin and ferritin (see Chapter XXIII, p. 656) and this effect might possibly be used as a method of differentiating various types of iron staining. Many modifications of the method have been used successfully to stain acid mucopolysaccharides ; two early ones were those of Hudack *et al.* (1949) and Ritter and Oleson (1950). The latter's method has been extensively used. Romanini (1951), Romanini and Giordano (1952) and Morone (1952) used Hale's method for studies on various mucopolysaccharides and the former authors regarded it as a refinement of the ferric chloride technique of Benazzi-Lentati (1941, 1942). Rinehart and Abu'l Haj (1951) introduced a method for making the reagent and claimed that with this the specificity for acid polysaccharides was increased. These authors, as well as Lillie and Mowry, noted that fewer tissue components stained when the pH of the reagent was lowered and in fact the pH of their reagent is below pH 2. Thus, provided the staining solution is strongly acid only SO_3H and PO_4 groups will be sufficiently strongly dissociated to bind ferric iron and, if nucleic acids are excluded (as is easily done) the specificity of the dialsyed iron for sulphated mucopolysaccharides becomes much higher.

Recently Abood and Abu'l Haj (1956) studied the interneuronal and intra-neuronal mucopolysaccharide in the sciatic nerve of bull-frogs and other species. They extracted from these by chemical means a substance regarded as non-sulphated hyaluronic acid. Histochemically the Hale method (Rinehart and Abu'l Haj modification) gave weak staining of the material in formalin-fixed paraffin sections. Treatment with testis hyaluronidase reversed this staining both in axoplasm and in the neurilemmal sheath. Ghiara (1954, 1956) compared Hale's reaction with the reaction of Benazzi-Lentati (1941, 1942) in which a dilute solution of ferric chloride (2-4 drops of a 10 per cent. solution in 10 ml. of distilled water) is applied to the tissues for 10 minutes and followed, after washing, by the Prussian Blue reaction. He obtained better results with Hale's technique and showed that raising the pH of the staining solution to pH 3 made the method much less selective. Lowering the pH or lowering the concentration of the colloidal iron both produced a greater sensitivity for strongly acidic polysaccharides.

Wolman (1956a), observing that at low pH levels only strongly acidic groups should be blocked by Hale's colloidal iron reagent, proposed to follow the full method with a method capable of demonstrat-

ing weakly acidic groups such as those of the uronic acids. For this purpose he found a positively charged colloidal gold solution to be satisfactory and the combined (Bi-Col) technique which he evolved is given in Appendix 9, p. 837. According to the original author this method (1) allows the differentiation of hyaluronic acid and CSA in skin, arterial wall, umbilical cord, etc. ; (2) it shows the presence of weakly acidic compounds in young cartilage, which is followed later by fixation of sulphate ; (3) it demonstrates intercellular material in the brain and a weakly acidic component in myelin and (4) it shows that a weak acid is secreted by the gastric parietal cells. I have applied this method to the study of the mucins of *Helix aspersa*, using cold microtome sections fixed briefly in acetic ethanol as well as paraffin sections after several special fixatives, and find that it is relatively easy to perform and that it gives a clear-cut distinction between blue and reddish-brown-stained materials. In my hands nearly all protein structures not stained blue appeared reddish-brown and it seems possible that the specificity of the colloidal gold part of the reaction for weakly acidic polysaccharides is low. Wolman (1956b) suggested that myelin contains an acid polysaccharide complex and he regards his findings with the Bi-Col method as supporting this concept. The blue interneuronal material which he demonstrated was identified as " ground substance." According to Wolman hyaluronic acid should and does stain brown by the Bi-Col method whereas according to Abood and Abu'l Haj it stains blue, albeit weakly. The latter authors' acceptance of hyaluronidase-lability as indicating the presence of hyaluronic acid is open to question. Since they were able to identify hyaluronic acid in their nerve extracts, however, and to show that this was not sulphated, the discrepancy between their results and those of Wolman cannot at present be resolved.

An interesting application of a modified Hale's reagent was reported by Berenbaum (1955) who obtained strong staining of the capsules of *Str. pneumoniae* and *Str. pyogenes* using saccharated iron oxide (ferrivenin, Benger) instead of colloidal iron. The majority of organisms showed a peripheral blue-stained layer which was more marked in the mucoid phase. Berenbaum's modification is applicable only to smears since the ferrivenin reagent stains all tissue components very strongly when used on tissue sections.

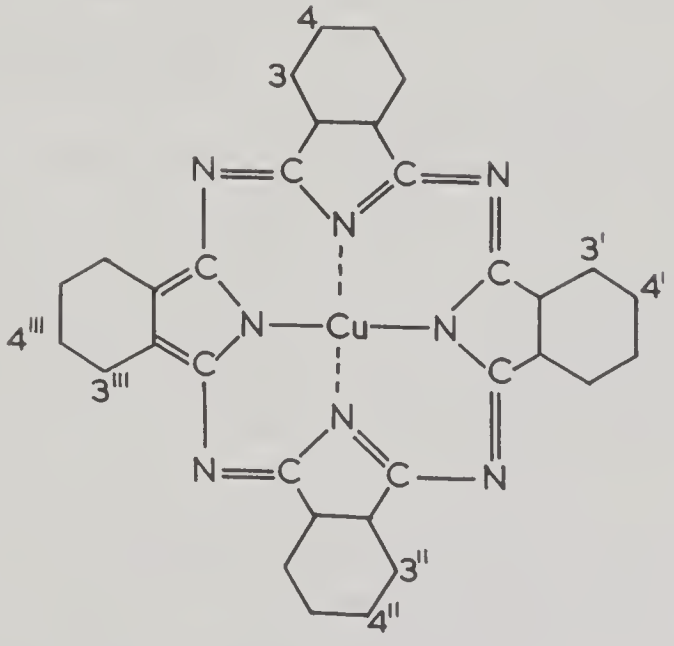
Hale's method was also modified by Muller (1955–56a) who substituted a solution known as " ferrihydrozydsol " for colloidal iron. This reagent is prepared by adding 12ml. of a 32 per cent. solution of $FeCl_3$ to 750ml. of boiling distilled water. A colloidal ferric hydroxide results which is stable for some months. Geyer (1956–57) compared this method with the original method of Hale and found that it produced a more distinct picture without blue colouring of the cytoplasm of

renal tubular cells. Nuclei, which stained by Hale's method, were unstained by the modification.

I conclude that although the specificity of Hale's method for acid polysaccharides, and for individual types within this group, is less precise than one would desire, the reaction is a useful one in applied histochemistry. In tissues such as brain, where a large part of the material is non-reactive (Plate IIIA, p. 289), a clear-cut demonstration of acidic polysaccharide can be obtained. Where most of the protein material is reactive it may be necessary to use a more dilute staining solution and to lower the pH. Especially in the case of fresh cold microtome sections (Figs. 77 and 84, pp. 257 and 284) these conditions produce excellent results and such sections are particularly recommended for use with the method.

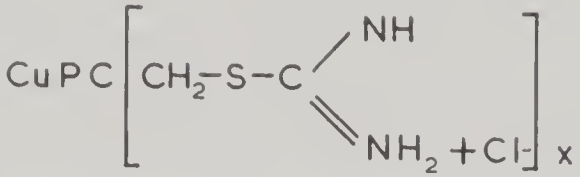
The Alcian Blue Method

The use of dilute solutions of Alcian blue 8GS as a specific stain for mucins was introduced by Steedman (1950). This author, using mainly material of other than human origin, showed that the dye possessed great advantages over other dyes for the staining of mucin, the chief of these advantages being its rapidity and ease of application. Alcian blue is a water-soluble copper phthalocyanin precursor of the insoluble dye Monastral Fast blue, into which it is converted by treatment with alkalis (Haddock, 1948). The copper phthalocyanins (CuPC) have a basic structure of the type shown below, and as such they are highly coloured water-insoluble pigments.



Various types of solubilizing groups are introduced into the molecule in the preparation of water-soluble dyes, the three most commonly employed being sulphonic acid, carboxylic acid and chloromethyl. It

is theoretically possible to substitute 1, 2, 3 or all four of the benzene rings in the molecule. The sulphonyl groups enter in the 3-positions but less is known about the positions taken by COOH and CH_2Cl. Alcian blue is recorded as belonging to a group of chloromethyl-substituted CuPC dyes in which the chloromethyl groups have been reacted with thiourea or alkylthioureas to give *iso*thiouronium derivatives which are very soluble in water.



According to the original author, Alcian blue did not distinguish chondroitin sulphuric acid complexes from mucoitin sulphuric acid complexes, and it therefore stained cartilage and mucin equally. In practice it was observed to stain acid mucopolysaccharides of epithelial and connective tissue mucin, but not stain the majority of muco-proteins. Its selectivity for mucins depended on the use of short staining; with longer staining nearly every tissue component became coloured. The original reaction, which was used by Vialli (1951) to demonstrate acidic groups after oxidation of tissues with chromic acid, was modified by Lison (1953) and Mowry (1956) by staining in acid solution. Lison used 0·5 per cent. Alcian blue in 0·5 per cent. acetic acid (30 minutes) and Mowry 0·1 per cent. of the dye in 3 per cent. acetic acid (30 minutes, pH 2·7 to 3·0). In fixed tissues and paraffin sections Alcian blue in acid solution stains connective tissue mucins and most epithelial mucins with usually negligible staining of the background proteins. In fresh tissues some of the nuclei stain even if the time of staining is kept to a minimum.

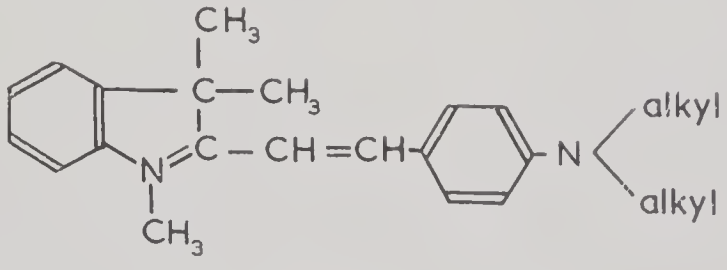
By the use of a strong (red) counterstain the staining of connective tissues by Alcian blue can be overlaid, leaving only the connective tissue mucins stained blue. This procedure is recommended for studying the latter and details are given in Appendix 9. Lison (1954) recommended chlorantine fast red 5B as a suitable counterstain ; others are tartrazine and neutral red or carmalum. Staining with an acid solution of Alcian blue can conveniently be followed by counter-staining with acid Solochrome cyanine R.S. (pp. 82 and 793). This combination gives brilliant nuclear detail and distinctive and different qualities of staining in the various mucopolysaccharides and muco-proteins. The use of Alcian blue is shown in Plate IIc, p. 289, where the highly sulphated mucins of the foregut of the lizard *Sceloporus undelatus* are seen to be brilliantly stained. Here the counterstain is neutral red. Another excellent counterstain is the PAS routine, with or without nuclear counter-staining.

Two alternative dyes of similar type to Alcian blue are Alcian green 3BX and Alcian green 2GX. The former stains bluish-green and the latter in a bright apple-green shade. The performance of the three dyes, as tested on paraffin sections of a variety of mucin-containing mammalian tissues and on unfixed and acetic-ethanol or alcoholic lead nitrate-fixed invertebrate tissues, is essentially very similar. Alcian green 2GX, however, sometimes stains known acid mucopolysaccharides in low concentrations when the other two dyes may fail. Alcian green staining of *Helix aspersa* mucins is shown in Fig. 78, p. 257. In Germany an equivalent dye of unpublished composition (Astrablau, Bayer-Leverkusen) has been used in place of Alcian blue for mucin staining. The recommended time of staining was 10 minutes in a 1 per cent. solution of the dye in 1 per cent. acetic acid.

The histochemical specificity of the stains described in this section has not been precisely determined. Staining with Alcian blue is prevented by prolonged methylation (Chapter V, p. 123) and greatly increased when acidic groups are introduced by sulphation or by oxidation with chromic acid or chromic acid following initial oxidation with periodic acid. There is little doubt about the mechanism of Alcian blue staining (by salt linkage with acidic groups) or that it can be made specific for the strongly acidic SO_3H group by lowering the pH of the staining bath to something below pH 2. In any case, the resistance of CuPC staining to bleaching, even by relatively violent procedures used as counterstains, constitutes an advantage over all other methods for acid mucopolysaccharides and it is safe to prophesy an increased use of the method in the future.

Experimental Cyanine Red

This cationic astrazone dye, produced by Dupont, was introduced by Quay (1957) for staining acid mucopolysaccharides and nucleic acids. Its formula



shows that it may be expected to behave rather similarly to Alcian blue. Quay advises its use as a 0·5 per cent. aqueous solution buffered to pH 2.9. After staining for 5–10 minutes the sections are differentiated in 3 changes of *n*-butanol before clearing and mounting. I have no experience of the method but the E^{max} of the dye (536mμ) compares unfavourably with that of the CuPC series (690–670mμ) so that unless

it proves to have some special qualifications as a mucin stain it will probably not replace the phthalocyanin dyes.

The Evans Blue Method

Gersh and Catchpole (1949) and later Catchpole, Gersh and Pan (1950) used intravital staining with Evans blue in their studies of connective tissue mucopolysaccharide-protein complexes (referred to in their terminology as glycoproteins). This dye forms a complex with plasma proteins and Catchpole and Gersh considered that it also formed a complex with the water soluble ground substance of connective tissue when this had been altered, by natural or other processes, to its more "fluid," depolymerized state. The method involved the injection of 1·25 per cent. Evans blue intravenously to rats (1 ml. per 100 g. body-weight) and killing the animals some 10 minutes later. In order to avoid loss of the water-soluble Evans blue-tissue mucopolysaccharide complex, fixation by freeze-drying was employed. According to these authors an "increase in depolymerization" of connective tissue mucopolysaccharides was also indicated by an increase in the degree of staining achieved by the PAS technique. If as in chondroitin sulphate the unit (disaccharide) is built up into blocks of n = 60 (1,3-linkage) only the two end glycols should be oxidized by HIO_4. If the linkage between the units is 1,3- or 1,2- no glycol is available for oxidation. If the linkage is 1,4-, periodic acid can cleave the glycol in the 2,3-position. Hydrolysis of bound CSA to individual disaccharides will therefore increase the amount of PAS staining provided the disaccharides also remain bound before and during oxidation.

Odd Methods for Polysaccharides

The Okamoto Method for Glucose

This method is based on the insolubility of Barium-glucose and Barium-lactose in alcohol. Thin slices of fresh tissue are fixed in methyl alcohol, saturated with barium hydroxide, for about 24 hours at 0–4° and, after processing, the precipitate of Ba-sugar is transformed into a silver precipitate. Gomori (1952), in quoting the method, felt that the accuracy of localization with this method was unlikely to be very high, but Muller (1955–56a and b) made experiments on filter-paper and concluded that the reaction should be capable of localizing glucose in the tissues. When the method was tested on mouse tissues a strongly positive reaction was obtained in the liver cells but not in the kidney.

It seems therefore that this method can be used in applied histochemistry and further applications of this principle, especially to fresh cold microtome sections, can be expected to produce further workable methods.

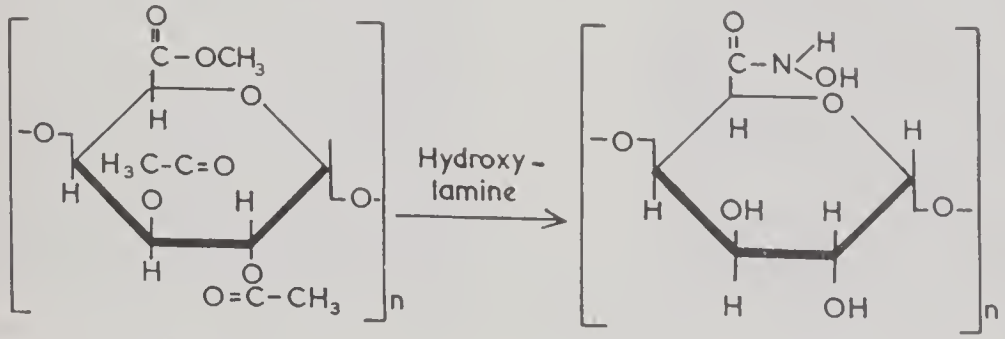
Methods for Pectin

The pectin group of polysaccharides is found in the cell walls and intercellular layers of all plant tissues (Whistler and Smart, 1953) and the main representative is galacturonan. This is a linear polymer composed of α-1,4′-linked D-galacturonoic acid units which may be fully or partially esterified. When not fully esterified the carboxyl groups are involved in salt formation with metallic ions, chiefly calcium and magnesium. The standard staining method for pectin, introduced by Mangin (1893) depends on its affinity for ruthenium red (ammoniacal ruthenium trichloride). Some workers have considered the method to have histochemical status and Carré and Horne (1927) showed that only pectic acid or pectates would give the reaction. When esterified, (pectin) pectic acid would not react. Bonner (1936) pointed out that the reagent would stain other substances containing free carboxyl groups and therefore most uronic acid-containing polysaccharides. Klein (1932) maintained that no specific reaction for pectin existed at that time.

Krajčinović *et al.* (1954–55) introduced a new reaction supposedly specific for pectins. This depends on the formation of an addition compound between pectic acid and benzidine. After diazotization of the free NH_2 group which remains at one end of the molecule an azo dye is formed by treatment with β-naphthol in alkaline solution. Details of this method are given in Appendix 9, p. 840.

Reactions for Acetylated Pectin

A reaction for the chemical estimation of acetyl groups in pectins was described by McComb and McCready (1957), based on earlier work by McCready and Reeve (1955). This depended on the production of pectin hydroxamic acids by treatment with hydroxylamine and the



I Acetylated Pectin

II Pectin Hydroxamic acid

$+ 2n\ CH_3CONHOH + n\ CH_3OH$

III Acetyl hydroxamic acid

formation of the red ferric complexes of II and III by treatment with ferric perchlorate.

This reaction can be performed histochemically on plant tissues though any colour in the sections must be due solely to pectin hydroxamic acid (II). It can also be carried out on mammalian tissues after acetylation of their polysaccharides in the usual manner. Fig. 83, p. 284, shows the positive reaction obtained in carbohydrate-protein compounds treated in this way. The specificity of the histochemical reaction, if any, remains to be determined for both plant and animal tissues.

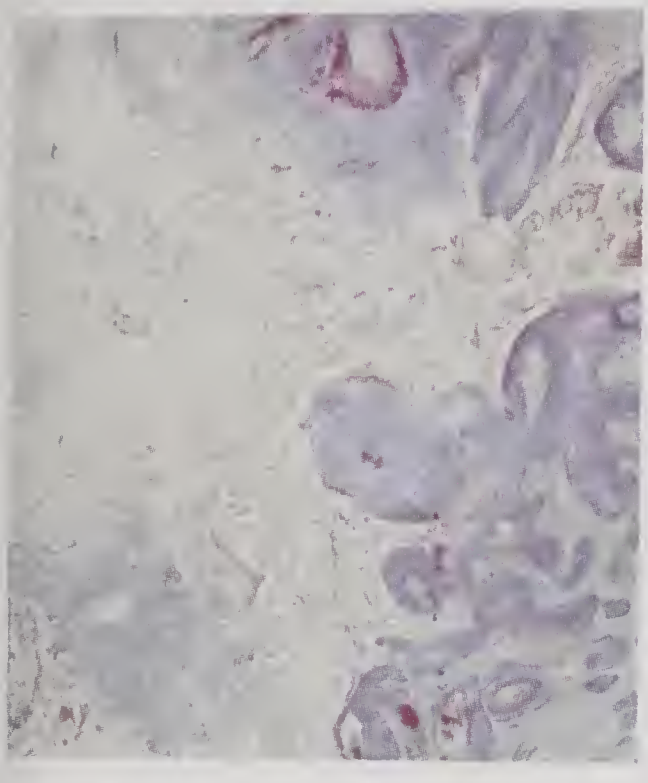
Germanic Acid Method for Polyvalent Alcohols

According to Feigl (1954) germanic acid, like boric acid, forms complex compounds with polyvalent alcohols such as glycerol, mannitol and glucose. Treatment of unfixed, cold microtome, sections with germanium dioxide in alkaline solution results in the formation of germanium complexes in materials (mucins) where hexose sugars are presumably present. After acid washing the presence of bound germanium can be demonstrated by means of an acid alcoholic solution of 9-phenyl-2,3,7-trihydroxy-6-fluorene. This forms a bright red absorption compound which is insoluble in water and alcohol and apparently stable. Fig. 79, p. 257 shows the result of this method applied to the mucins of the snail *Helix aspersa*.

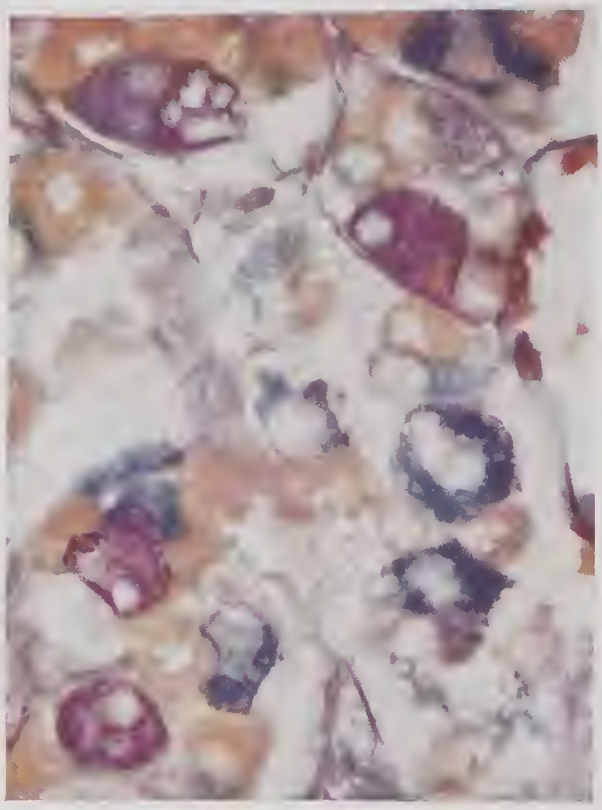
Methods for Chitin

The occurrence of this polysaccharide has already been mentioned (p. 229 above). The oldest method for demonstration of the various chitins employs the Schultze, chlor-zinc-iodine reagent after treatment with warm KOH (50 per cent.) and washing with alcohol. Chitin stains reddish violet. This reaction works much better in the test tube than under histochemical conditions. Lison remarks (1953) à propos such methods that " les réactions de la chitine . . . n'étaient guère commodément réalisables à cause de leur brutalité." A better variation (Schultze, 1922) depends on prior treatment of sections with diaphanol (chlorodioxyacetic acid) followed by the chlor-zinc-iodine reagent (Iodine 6·1 g; KI 10 g; zinc chloride 60 g; distilled water 14 g.). The author suggests that diaphanol removes incrustations from the chitin and allows it to react with his reagent. Lison, however, does not accept this since many chitins are not so incrusted. Chitin stains violet, as does cellulose (filter-paper), with which the reagent should be tested before use.

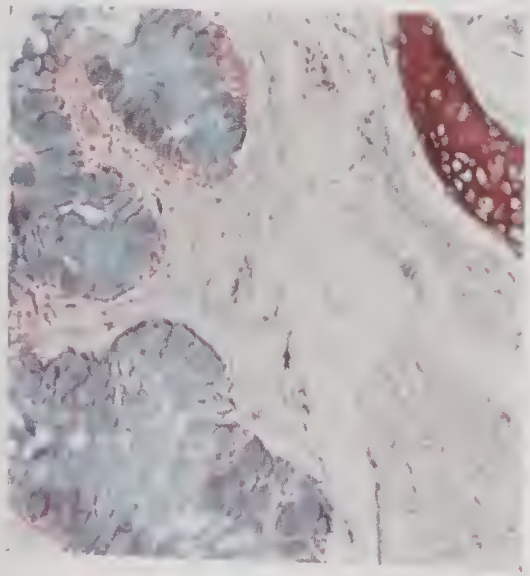
Although it is a grossly unspecific method the alkaline tetrazolium reaction (Chapter V, p. 114) gives a blue or bluish-purple reaction with all varieties of chitin on which I have tested it. Like the diaphanol-



IIA. Mouse cervix. Methylcholanthrene-indueed squamous cell careinoma. Growing edge of the tumour. Solochrome cyanine RS. × 124.



IIB. Human adenohypophysis. Distinction between R-cells (magenta), S-eells (blue) and aeidophils (orange). Performic aeid—Alcian blue, PAS-orange G. × 870.



IIC. *Sceloporus undelatus* (lizard), foregut. Blue staining of sulphated epithelial mucins. Alcian blue—Neutral red. × 65.

IID. *Triton rubicundus* (whelk) etenidium. The gill plates stain blue over the outer two-thirds of their length. In the absence of SH groups and reducing lipids this reaction indicates the presence of reducing sugars (chitin). Alkaline tetrazolium reaction. × 270.

[*To face p.* 264.

Schultze method it has also the merit of leaving sections intact. Chitin which has been tanned does not give a positive reaction but chitin which is free from phenolic substances (and also from SH groups and reducing lipids) reacts strongly. Plate IIp shows a positive reaction in the ctenidium of *Triton rubicundus* in which the gill plates stain deep blue at one end, tailing off to a fainter blue at the other end. As an alternative to the above methods chitin can be effectively sulphated by one of the methods suggested by Kramer and Windrum (1954) and given in Appendix 9, p. 835. The resulting chitin sulphate can sometimes be demonstrated by the metachromatic reaction or better by staining with an acid solution of one of the Alcian dyes (pH 3·6).

Alkaline DMAB Method for Amino-sugars

It was observed by Ehrlich (1901) that certain mucins and mucoids gave a purple colour when treated with warm solutions of dilute alkali and subsequently with *p*-dimethylaminobenzaldehyde. Aminoff *et al.* (1952), described the reaction as characteristic of *N*-acetylhexosamines and they showed that it depended not only on the pH at which the reaction was carried out but also on the nature of the buffer. The strongest reaction was observed with borate buffer at pH 9·8.

This reaction can be applied histochemically but the colours given by the *N*-acetyl sugars of the majority of mucins are very weak. The reaction is negative with insect chitin and it cannot be recommended as an alternative to the reactions given below.

Methods for the Demonstration of Glycogen

The action of ptyalin (saliva) or diastase, at room temperature for 30 minutes or less, is considered sufficient to remove glycogen from paraffin sections whatever the fixative employed. It is known that both formalin and picric acid may render glycogen insoluble in water by binding it to protein, but there is no suggestion that it is made impervious to the action of diastase. The concept of glycogen being bound to protein, and therefore less easily extractable, led Willstätter and Rohdenwald (1934) to describe a complex called desmoglycogen. Meyer and Jeanloz (1943) have proved that glycogen is not chemically bound to protein, but, after mechanical inclusion in coagulated protein, glycogen of high molecular weight cannot be extracted by washing in hot water. This bound fraction is considered by Prins and Jeanloz (1948) to be the equivalent of Willstätter's desmoglycogen.

There is no doubt that glycogen present in fixed sections of skin, liver, muscle and other tissues is always entirely removable by saliva or diastase. The latter is more certain in action, but there is evidence that it attacks a wider range of substances. At the present time it is accepted that material remaining in sections after the use of diastase,

however it may react, is not glycogen. Some difficulties have arisen over the interpretation of the reaction in the case of the leucocytes. The strongly positive PAS reaction in the cytoplasm of human polymorphonuclear leucocytes and megakaryocytes has been ascribed by Wislocki, Rheingold and Dempsey (1949), and by Gibb and Stowell (1949), to the presence of glycogen, removable by salivary digestion. I have found, however, that a considerable amount of PAS-positive material remains in human polymorphs and megakaryocytes, both in Susa-fixed blood smears and in formalin-fixed paraffin sections, after the prolonged action of saliva or diastase ; moreover, Wislocki and his collaborators agree that in the megakaryocytes of the monkey (*Macaca mulatta*) the PAS-positive material is not glycogen. These findings could be due to the presence of carbohydrate-containing substances other than glycogen, to the presence of lipid substances of the phosphatide class, or to incomplete removal of glycogen due to the presence of a protein-bound, diastase-fast fraction. Traces of lipid present as lipoprotein complexes in paraffin sections could not be responsible for the reaction and incomplete removal of glycogen after treatment with diastase has not been recorded. It is likely that the material demonstrated is a carbohydrate-protein complex.

Recent conceptions of the chemical and physical structure of glycogen have indicated that there are a number of naturally occurring glycogens with different degrees of polymerization and hence with different solubilities (Meyer, 1943). This knowledge has partly explained the difficulties in histological fixation of the glycogens and this aspect of the matter has been dealt with in Chapter IV ; it has made no difference to the demonstration of glycogen remaining in tissue sections. There are still five basic methods in use for this purpose and all of them have some merit. Whichever method is used for demonstrating glycogen control sections must always be employed. These are incubated with saliva (Bauer, 1933 ; C. M. Bensley, 1939), or preferably with malt diastase (Lillie and Greco, 1947), which removes the glycogens, and they are then treated in the same manner as the unincubated sections. Lillie and Greco advised the use of 1 per cent. malt diastase in saline phosphate buffer at neutrality, but this refinement is unnecessary since 1 per cent. diastase in 1 per cent. saline, or even in distilled water, is sufficiently active to remove glycogen from sections fixed in all ordinary fixatives in 30 minutes at room temperature. Bunting and White (1950) used 0·2 per cent. diastase in M/60 potassium dihydrogen phosphate (pH 5) at 37° for 2 hours, and compared their diastase control sections with sections treated in buffer alone. Such a precaution is unnecessary if one uses the aqueous solution. If 1 per cent. saline is used, even with the short incubation employed, substances other than glycogen will be removed from the

section. Since these substances (RNA, for instance) are unstained by procedures for demonstrating glycogen, no error is introduced by this circumstance.

Iodine Methods

The staining of glycogen with aqueous solutions of iodine, though certainly the oldest method, is still employed in modern histochemistry. The technique was used by Claude Bernard (1877) in his classical researches on the liver and also by Paul Ehrlich (1883) for the demonstration of glycogen in diabetic livers. Solutions of iodine in potassium iodide (such as Lugol's) are usually employed and the main practical difficulty is the production of permanent sections. Lison (1936) gave a selection of three methods designed with this object. That of Langhans (1890) modified by Carleton (1938) employed dehydration of paraffin sections in saturated iodine in absolute alcohol, followed by clearing in essence of origanum, xylene, and mounting in balsam. Gage's (1917) method used fixation in acetic alcohol containing iodine. Paraffin sections were subsequently treated with an alcoholic iodine solution containing potassium iodide, without removal of the paraffin. After drying, the sections were deparaffinized in xylene and mounted in balsam. Driessen (1905) fixed his material in alcohol and stained paraffin sections with phenolic xylene containing Lugol's iodine, vigorously agitated at the time of preparation. Subsequently the sections were cleared in phenolic xylene and in xylene before mounting in balsam. Of the three methods mentioned, Carleton's is probably the best. Iodine methods have the drawback that their specificity is not very high, since a similar brown colour is produced by various protein constituents and by amyloid. The use of saliva or diastase controls removes this objection. Mancini (1944) used the iodine method to demonstrate glycogen in cartilage cells fixed by the freeze-drying method.

The Bauer-Feulgen Method

The original method described by Bauer (1933) employed hydrolysis and oxidation of the polysaccharide glycogen with 4 per cent. chromic acid for a short period, and demonstration of the resulting polyaldehydes with Schiff's solution. This method has certain disadvantages which preclude its use for accurate work although it is still used in modern histochemistry (C. M. Bensley, 1939 ; Bunting and White, 1950). The chief disadvantage is the non-specific colouring occurring in various tissue components which do not contain glycogen. Among these mucin and thyroid colloid, which certainly contain no glycogen, have been shown to react strongly (Wallraf and Bechert, 1939 ; Bignardi, 1940 ; Dempsey and Wislocki, 1947). Although the use of

diastase controls reduces this objection it remains true that small amounts of glycogen are difficult to appreciate by Bauer's method, which also suffers from the non-specific staining of plasmalogens, inherent in the use of Schiff's reagent, in the same way as do the Feulgen nucleal reaction and the PAS technique. Although good histological results can be obtained, the method is no longer to be recommended in histochemistry.

Silver Methods

Several methods (all comparatively recent) have been devised for the demonstration of glycogen by means of silver complexes. They all employ acid hydrolysis and oxidation to reveal aldehyde groups in the polysaccharide chain prior to their combination with silver. Silver methods have the advantage that the end product is metallic silver which, being black, is easily visible under the microscope and is especially easy to photograph. The methods so far evolved may be divided into two classes, depending on whether or not additional reduction by means of formalin follows the silver bath. In the first group, in which formalin reduction is employed, the first to be described was the method of Mitchell and Wislocki (1944). These authors used acid potassium permanganate as oxidizing agent followed by bleaching in alcoholic oxalic acid-silver nitrate before treatment with ammoniacal silver oxide, and formalin. A second method in this group was that described by Arzac (1947) who also used $KMnO_4$, followed by ammoniacal silver carbonate and formalin reduction. Gomori (1946) criticized the method of Mitchell and Wislocki and his objections apply equally to the method of Arzac. He considered that Mitchell and Wislocki's method was unselective because the correct pre-treatment for the liberation of aldehyde groups was not employed and because the employment of formalin as a reducing agent stained collagen and reticulin as well as specific polysaccharide. Gomori introduced a different technique employing hydrolysis and oxidation with chromic acid (5 per cent.) as in the Bauer method. He did not advance any reason for regarding chromic acid, rather than any other oxidizing agent, as the correct method for liberating aldehydes. Hydrolysis was followed by staining in a solution of a hexamine-silver nitrate complex buffered to alkaline pH with sodium borate. By this method, which belongs to the second group of silver methods for glycogen, mucin also stained strongly.

For all these three methods collodion protected slides were recommended since a thin film of this material prevents loss of glycogen from the sections which otherwise occurs during hydrolysis and oxidation. As Lillie and Greco (1947) have shown, in the case of diastase control sections, collodion must be applied after and not before incuba-

tion with the enzyme, since it prevents the action of the latter to a variable extent. Arzac and Flores (1949) investigated the different effects of varions reagents on the staining of glycogen by silver methods. They found that 10 per cent. chromic acid (20–30 minutes) was the best but specifically excepted periodic acid which was not available to them at that time. Lillie (1948) showed that this reagent could precede staining with silver complexes in the demonstration of glycogen. Arzac and Flores also studied the effect of formalin and of the type of silver solntion employed. Of the latter, ammoniacal silver carbonate was found easy to prepare, stable, and rapid in action. In order to increase the rate of reduction of silver the solutions were nsed at 50° (for 1 hour). Gomori's hexamine-silver and a piperazine-silver complex were also tried bnt found to be slower in action than the ammoniacal carbonate. Formalin reduction was found to widen the range of substances giving a positive reaction, independently of the type of hydrolysis and silver complex employed. Reticulin was invariably stained. However, sharper images and quicker results were obtained in the case of glycogen.

It must be obvious that Gomori's (1946) objection to formalin reduction is a valid one if the silver techniques for glycogen are to be regarded as having histochemical significance. Gomori's original method is recommended and this and the two solutions evolved by Arzac and Flores to take the place of hexamine-silver appear in Appendix 9.

The Periodic acid-Schiff Method

The PAS method reveals glycogen particularly clearly and it has been used for this purpose by Marchese (1947), Wislocki, Rheingold and Dempsey (1949), Gibb and Stowell (1949), and by McManus and Findley (1949), among others. Although it was with the specific idea of preserving glycogen in the tissues that Hotchkiss introduced his buffered alcoholic solutions there is no doubt that these reduce the rate of HIO_4 oxidation so much that part of the glycogen bound in the tissues is not stained afterwards by fuchsin-sulphurous acid. It is therefore essential to use watery solutions of HIO_4, preferably on paraffin sections which have been protected by a thin layer of collodion. The method is less satisfactory for formalin-fixed frozen sections, freeze-dried sections and cold microtome sections.

The Lead Tetra-acetate-Schiff Method

This method, introdnced by Shimizu and Kumamoto (1952) as a substitute for the PAS reaction, appears to possess no advantages over the latter for the demonstration of glycogen. Casselman (1954) found that the demonstration of glycogen by this method was dependent

on the conditions of oxidation. If the $PbAc_4$ was dissolved in anhydrous solvents no staining occurred. If aqueous sodium acetate, or water alone, were present, strong reactions were obtained. The reaction is not recommended, however, for the demonstration of glycogen.

Best's Carmine Method

Although the use of such an empirical method cannot be reconciled with the strict histochemical point of view, the ammoniacal carmine solution of Best (1906) cannot be dispensed with. It remains in current use by histochemists as well as by histologists (Lison and Vokaer, 1949). According to Baker (1945), the active principle of the natural dye carmine is carminic acid which, at a pH on the alkaline side of its isoelectric point (4·0–4·5), is negatively charged and behaves like an acid dye. Although we know that the carminic acid in Best's solution is present in this form, its staining of glycogen depends on physical and stereochemical factors which at present we do not understand. It is not necessary to use celloidin-embedded sections as originally advised by Arnold (1908) but paraffin sections should preferably be coated with a thin film of celloidin before they are transferred to Best's solution. Alternatively, double-embedding of tissue blocks in celloidin and paraffin can be employed. Mullen (1944) described a rapid method of staining glycogen with Best's solution using alcohol-fixed paraffin sections and covering with celloidin in the above-mentioned manner. Differentiation may be carried out with Best's differentiator (aqueous methyl and ethyl alcohols) or simply with absolute methyl alcohol.

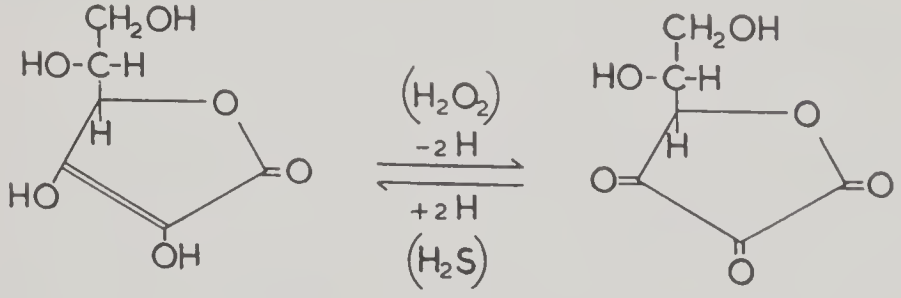
Opinions as to which of the methods listed above is the most suitable vary considerably. A recent evaluation of the various methods by Carpenter, Polonsky and Menten (1951) stressed the difficulty of ascribing specificity for localization of glycogen to any of them, on account of the large number of factors operating against such specificity. For routine use, I consider that celloidinized paraffin sections, fixed in whatever fixative is habitually used, should be stained with Best's carmine. Control diastase-treated and subsequently celloidinized sections should be employed in parallel. If an accurate study of the glycogen distribution in any particular tissue is proposed, some improved fixation such as that suggested by Lison and Vokaer (Chapter IV), followed by Best's stain, or by PAS, is the most suitable combination. The iodine methods will not generally be employed because of the difficulty of obtaining good results with ordinarily fixed material and of obtaining permanent mounts ; their only real use is for freeze-dried material and for fresh frozen cold microtome sections. The silver methods, which have considerable advantages for photography, are not otherwise especially to be recommended, and of the methods depending on Schiff's reagent, oxidation by periodic acid is to be

preferred to oxidation by chromic acid. The PAS method, using celloidinized slides after Lison-Vokaer fixation, or other fixation in cold alcoholic mixtures, is considered by many workers to give better results than similar preparations stained with ammoniacal carmine. The choice will depend upon the tissue under review and if this contains PAS-reactive substances, mucopolysaccharides, mucoproteins, glycoproteins or unsaturated lipids, in the same situation as the glycogen, then the carmine method, despite its empirical nature, may give better results.

The Demonstration of Ascorbic Acid

Theoretical Considerations

For the demonstration of ascorbic acid in the tissues only one recognized method exists. This is the acid silver nitrate technique of Bourne (1933a and b, 1936). Attempts have been made to transfer methods of biochemical estimation to the histochemical demonstration of the substance (Giroud *et al.*, 1939) but these have not been successful. Bourne's original method was based on the observation by Svirbely and Szent-Györgi (1933) that vitamin C in the adrenal cortex would reduce silver nitrate solutions, and the very similar method of Giroud and Leblond (1934, 1935a) was also based on this fact. The reduction of acid silver nitrate without the action of light or heat was thought to be specifically the property of ascorbic acid. However, ascorbic acid may be present either in reduced or oxidized form, and it is only the former that will give a positive reaction with acid silver nitrate. Efforts to obviate this have been based on pre-treatment of the fresh frozen sections with H_2S, by saturating their container with this gas and by subsequently exposing the tissues to a vacuum in order to remove the excess of H_2S not used in the process. The following reaction takes place :



L-Ascorbic acid (reduced form)

dehydroascorbic acid (oxidized form)

From the outset strong criticism was levelled at the specificity of the acid silver reaction. Harris and Ray (1933) drew attention to the fact that in the liver and adrenal medulla, both rich in ascorbic acid by chemical estimation, no black deposit of silver was produced by the

Bourne technique. Glick (1935) also doubted the specificity of the reaction and used instead a refinement of the 2 : 6-dichlorphenolindophenol method of Birch, Harris and Ray (1933), upon tissue sections 20 μ thick cut with a rotary microtome. With this technique Glick and Biskind (1935, 1936a, b and c) and Biskind and Glick (1936) made a study of the distribution of ascorbic acid in the anterior and posterior hypophysis, corpora lutea, small intestine, thymus and adrenal. These studies have been continued, using refinements of the same technique, by Glick *et al.* (1953) and by Bahn and Glick (1954). King (1936) likewise maintained that silver nitrate was reliable neither as a qualitative nor as a quantitative index for vitamin C in the tissues. On the other hand, Giroud and Leblond (1936) defended the specificity of the silver nitrate test and claimed that negative reactions in scorbutic tissues could be reversed in a few hours by the administration of ascorbic acid. A positive reaction, according to these authors, could be taken as an indication of the presence of ascorbic acid while a negative reaction, which might be due to factors inhibiting the reduction of silver nitrate, did not necessarily indicate absence of ascorbic acid.

Barnett, Bourne and Fisher (1941), while using the acid silver nitrate technique to study developing bone, observed a heavy deposit of silver in the trabeculæ which blackened by the action of light during further preparation of the sections. These authors found that this type of silver precipitation, which differed morphologically from the small granules produced by ascorbic acid, could be removed by subsequent treatment of the tissues with 5 per cent. ammonia for 10–15 minutes in the dark. The inclusion of this modification was therefore considered to increase the specificity of the reaction. Further criticism came from Bauer (1943) who concluded from his experiments that either vitamin C granules could be demonstrated by a variety of methods or that silver granules could be produced in the cytoplasm by the acid silver technique which had no relation to ascorbic acid. Barnett and Fisher (1943) conducted a series of experiments with the staining of mixtures of gelatine, ascorbic acid, and olive oil or ground glass, by the usual silver method. They concluded, despite their earlier work, that it was unjustifiable to infer the whereabouts of ascorbic acid from the situation of silver precipitate obtained by the method, and that the precipitation of acid silver nitrate took place at various interfaces. The tendency of the Golgi apparatus to stain by the method was explicable on this basis. Bourne (1944, 1950), however, continued to support the specificity of the method partly on his own (1933a) evidence that the disposition of mitochondria in the pituitary and adrenal stained with Janus green was identical with the distribution of silver-stained granules and partly on the work of Giroud (1938) who found complete similarity in the morphology and dimensions of mitochondria and of

the silver-stained granules. Chayen (1953) also found ascorbic acid in the mitochondria in his studies on plant tissues and he introduced a number of modifications designed to increase its specificity. Jensen and Kavaljian (1956), however, found that in *Allium cepa* and *Vicia faba* the silver deposits appeared as cytoplasmic particles 0·2 to 0·6 μ in diameter. These were localized predominantly in the vicinity of the cell walls, in the root tip cells and also in the elongating cortical parenchyma.

Lison (1953) maintains that there is no doubt that the cytochemical localization of ascorbic acid by the technique of Giroud and Leblond corresponds to that given by chemical estimations. He does not believe, however, that the intracellular localization can be regarded as precise. Clara (1952, 1953) gives a list of materials other than ascorbic acid which are present in cells and tissues and which react with the acid $AgNO_3$ reagent. Amongst these are melanin, adrenochrome, enterochromaffin substance, pancreatic α-cell granules and granules of neurosecretory substance in the hypothalamus. Hagen (1954) carried out estimations of ascorbic acid in particulate and non-particulate fractions prepared by ultracentrifugation of sucrose dispersions of ox adrenal and dog liver. He found that ascorbic aeid was entirely or almost entirely in the non-particulate fractions and concluded that the localization of reduced silver particles could not indicate the localization of ascorbic acid within the cell. Eränkö (1954) made a number of experiments to check the reliability of the method. He found that a clear solvent zone diffused into fresh adrenal tissue at a higher speed than silver nitrate and suggested, for this reason, that sections should be much better than slices. Certainly freeze-dried sections, as used by Eränkö, are excellent for the purpose and fresh cold microtome sections can be used equally well. Ascending paper chromatography was carried out by Eränkö with 30 μ adrenal sections placed on the paper at varying distances from the base line. In every case at least 20 per cent. of the ascorbic acid in the sections diffused out with the solvent front before the $AgNO_3$ arrived. He agreed with Bourne (1950), however, that reduction of $AgNO_3$ by ascorbic acid is so rapid that once reduced, diffusion of the silver precipitate would not occur. It is evident, however, that even with thin sections mounted on slides or coverslips some diffusion will take place before this event occurs.

Application of the Silver Nitrate Technique

There are two principal ways of applying the acid silver nitrate technique for ascorbic acid : (1) by application of the reagent to unfixed frozen sections, and (2) by fixation of freshly excised blocks of tissue with alcoholic acid silver nitrate. Deane and Morse (1948) found this last variant to be the most successful, and it was also

employed by Bacchus (1950), who placed rat adrenal glands in a dark vial containing 5 per cent. $AgNO_3$ at pH 2 to 2·5 within 1 minute of extirpation. This author found a reducing substance present in nearly all the cells of the cortex which he considered mainly attributable to ascorbic acid. Two types of cortical cells were recognized, one with "ascorbic acid" granules aggregated peripherally close to the cell membrane, the other with aggregation in the perinuclear region. Whichever of the above variants is employed, it is possible to produce small black silver-stained granules in a wide variety of tissues, not only in the cells but also in tissue spaces and in the connective tissues. Macrophages in the supporting tissues of the adrenal cortex and pituitary gland constantly appear full of granules by the acid silver method.

It is not necessary, therefore, to decide whether the method works, but whether its specificity for ascorbic acid is absolute, relative or doubtful. Several substances besides ascorbic acid are known to reduce acid silver nitrate, the phosphate deposits observed by Barnett, Bourne and Fisher (1941), melanin granules (Bizzozero, 1908 ; Giroud and Leblond, 1936) and the various materials mentioned by Clara (1952) which have been listed above. Phosphates will obviously give little trouble in most tissues and in any case they are prevented from interfering in the final result by the modification introduced by Barnett *et al.* Melanin granules, similarly, cause little confusion. There is a significant common factor present, however, in three of the tissue components which are reported as positively staining with acid silver in the fresh state. These components are the mitochondria (Giroud and Leblond, 1935b), the Golgi apparatus (Hirsch, 1938) and the large granules of the adrenal and pituitary macrophages. All of these contain lipid, which is probably phosphatide. It has been shown by Alsterberg (1941) that phosphatides will cause the precipitation of silver from silver nitrate solutions and that the optimum pH for this reaction is in the acid range. There is no direct evidence that such lipid substances are responsible for the ascorbic acid reaction and these speculations do not exclude the possibility that, as the chief supporters of the reaction contend, ascorbic acid is present in all three situations. They do give rise to legitimate grounds for suspicion that the method has low specificity and that accurate intracellular histochemical localization of ascorbic acid by its means is not possible. Danielli (1946) put forward a reasoned criticism of the specificity of the method with respect to localization. According to his view the ascorbic acid and silver ions and probably their initial reaction products are highly diffusible. Consequently one would expect the silver precipitate, if localized at all, to be in the mixing zone in the cell cortex if the reaction product is indiffusable and on any suitable adsorbing surface if it is

diffusible. For these reasons he considered that the acid silver technique could not show the *in vivo* distribution of ascorbic acid unless this was fixed chemically to an "indifferent body," and either the reaction product was not diffusible or else had a high affinity for fixed ascorbic acid. It is possible that these conditions, especially the first, may be fulfilled, but they have not been shown to exist by experimental means.

Since no other technique exists, the frozen section method of Bourne, together with the modification of Barnett *et al.* and the block staining technique by Bacchus (1950), appear in Appendix 9.

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CHAPTER X

APPLIED MUCOPROTEINS (AMYLOID)

The removal of the problem of amyloid to a separate chapter is not intended to suggest that it is of greater importance than those polysaccharide-containing protein components of the tissues discussed in Chapter IX and elsewhere. Histochemically amyloid does not readily fit into the applied proteins of Chapter VII or into the polysaccharides of Chapter IX. Hence its isolation in a separate chapter. It would be logical to consider here also the applied histochemistry of the mucins. At the time of writing, however, it must be said that this much neglected subject does not warrant any further attention than that given already in Chapter IX.

The History and Occurrence of Amyloid

The deposition of a hyaline homogeneous substance in the walls of blood vessels and in the connective tissues was observed by many workers before it was described by Rokitansky (1842) under the comprehensive term lardaceous disease. Virchow (1860), in 1853, named the substance amyloid because of its starch-like behaviour when stained with iodine. Virchow clearly regarded amyloid as a carbohydrate-containing substance, and the much later studies of Eppinger (1922) and Hass (1942) showed that it was indeed a carbohydrate-containing protein. Virchow considered that pre-amyloid in the circulating plasma was transformed into a gel after crossing the vessel walls. Nothing has occurred in the last 100 years which radically alters this historic conception.

The deposition of amyloid is usually divided into primary and secondary amyloidosis on the basis of the presence or absence of an exciting cause for its production and on the normality or otherwise of its distribution and on its staining characteristics. Primary amyloid, for instance, almost invariably involves the heart and in two-thirds of the cases the liver, kidney, spleen and adrenals are not affected. In the secondary form of amyloidosis the heart is free from deposits, but liver, spleen, adrenal and kidney are invariably involved. The affinity of secondary amyloid for Congo red and methyl violet is usually considered to be high, but primary amyloid may react weakly or not at all.

Amyloidosis has long been considered a disorder of protein metabolism because it is characterized by the abnormal deposition of

protein complexes in various organs. Several observations have suggested that amyloid deposits are at least partly derived from proteins circulating in the blood under abnormal conditions (Richter, 1956). It has been shown by Luetscher (1940) and by Johansson (1949), as well as by a number of other authors, that the serum protein pattern is abnormal in amyloidosis and in multiple myelomatosis abnormal levels of serum globulin and amyloid coexist. Moreover, the incidence of amyloidosis is high in hyper-immunized horses used for the production of antisera. Castillo (1935) reviewed very thoroughly the relationship of protein metabolism to the precipitation of amyloid and this was considered also by Teilum *et al.* (1951) and by Pirani (1951). A comparative study of amyloid deposits in several different species was made by Hass, Huntington and Krumdieck (1943).

The most interesting hypothesis of the cause of amyloidosis is that of Teilum (1956), which is the result of a long series of experimental and pathological studies on the subject. This author believes that amyloid protein is synthesized by mesenchymal and reticuloendothelial cells in excessive quantities when the normal mechanism controlling this function ceases to operate. It is postulated that persistent antigenic stimulation and other forms of chronic stress may cause breakdown of the controlling mechanism. This would account for the very high incidence of histologically demonstrable amyloid deposits in rheumatoid arthritis (Reece and Reynolds, 1954; Teilum and Lindahl, 1954).

The role of the plasma cell in amyloid production was first suggested by Teilum (1948), who postulated that it was responsible for the abnormal production of globulin in allergic states. My own studies (Pearse, 1949) on plasma cells containing the so-called Russell bodies showed that these consisted for the most part of mucoprotein and that this mucoprotein was manufactured by the cells rather than absorbed from elsewhere. It has been suggested by Lundin *et al.* (1954) that plasma cell proliferation is a general reaction promoted by a pituitary factor which Schelin *et al.* (1954) have identified with growth hormone (somatotrophin). It had previously been shown by Cavallero (1953) that injection of this hormone into rats produced plasma cell proliferation. There is little doubt that the plasma cell and other mucoprotein-secreting reticulum cells are mainly responsible for production of the protein (mucoprotein) component of amyloid.

Artificial Production of Amyloid

Several techniques have been employed for the production of amyloidosis in animals. In mice, feeding with a casein-rich diet or repeated injections of casein have been used successfully by a number

of workers and injections of ribonucleate have also been employed. The latter method was first used by Letterer (1926, 1934), and the production of amyloid was attributed to the formation of abscesses which were produced by the unsterile RNA injections. Goessner *et al.* (1951) used a similar method for the preparation of experimental amyloidosis. Richter (1954), however, produced amyloid in rabbits with buffered RNA passed through a Seitz filter before use. Injections of Freund's adjuvant were used by Rothbard and Watson (1954) to produce artificial amyloid by stimulating the production of antibodies to various antigens. Uotila, Peräsolo and Vapaavuori (1955) tested the effect of intercurrent injections of somatotrophin and TSH on caseinate amyloidosis in mice. They found that TSH caused a rise in production of amyloid, but that somatotrophin was without effect. This observation is not in keeping with some of the findings reported earlier in this chapter, where somatotrophin was found to stimulate plasma cell proliferation.

Protein Patterns in Amyloidosis

A detailed study of the electrophoretic patterns of sera in cases of amyloidosis was made by Bohle, Hartmann and Pola (1950). These authors found a transient rise of α and β-globulins followed by a more persistent rise in γ-globulins. Wagner (1955) investigated two cases of amyloid and found in one a raised serum α1-globulin fraction associated with a mucopolysaccharide and in the second raised α2- and β-globulin fractions, which were again associated with an abnormal mucopolysaccharide. Changes in the plasma proteins had been considered non-specific by Letterer and Schneider (1953), but Wagner's findings clearly suggest otherwise. They suggest, furthermore, that association of the protein or mucoprotein (globulin) component of amyloid with the mucopolysaccharide component may take place in the bloodstream rather than at the site of deposition.

Studies of the amino-acid content of serum protein, liver protein, amyloid, hyaline and collagen were made by Letterer, Gerok and Schneider (1955). These showed that tyrosine, absent from hyaline, was present in moderate amounts in amyloid. Tryptophan was unfortunately not measured. The total number of lipophilic groups (Gly. Ala. Val. Leuc.) was comparatively high, but the number of basic groups was lower than might have been expected. Figures for cystine and methionine were similar to those published elsewhere for human γ-globulin, but for most of the other amino-acids the relationship was not particularly close. In a sample of human liver amyloid Giles and Calkins (1955) found 1·5 per cent. of hydroxyproline, 3·8 per cent. of glycine and 4·1 per cent. of tyrosine. They concluded that less than 10 per cent. of the dry weight of their material was composed of

collagen. Other tests showed that their sample of amyloid contained 82·8 per cent. of water, indicating the presence of a hydrophilic protein. The total carbohydrate content was over 4 per cent. of the dry weight, 1·86 per cent. being neutral sugar, 1·55 per cent. hexosamine and 0·6 per cent. hexuronic acid. It is not possible to tell from these figures what percentage of the carbohydrate content was to be ascribed to internal components of the mucoprotein and how much to associated mucopolysaccharide. Treatment of amyloid with 5 per cent. HCl in methanol at 100° enabled Klenk and Faillard (1955) to extract from it the methylglycoside of neuraminic acid (see Chapter XI, p. 293).

The Histochemistry of Amyloid

Protein Tests. As might be expected from the results of amino-acid assay, histochemical methods for the basic amino-acids give only weakly positive results. Tests for basic protein, such as chromotrope 2R at pH 2 (Wagner, 1957) or acid Solochrome cyanine, are also weakly positive. Although sulphur-containing amino-acids are present in amyloid, tests for these are negative or at most faintly positive. On the other hand, histochemical tests indicate the presence of a considerable quantity of tryptophan in samples of human secondary amyloid and this has not been recorded chemically. The application of the DMAB-nitrite method (Chapter V, p. 93) to an unfixed cold microtome section of kidney containing amyloid deposits is illustrated in Fig. 80. This indicates, at a rough estimate, that amyloid contains about the same amount of tryptophan as fibrin.

Congo Red. The most popular simple staining method for amyloid is the Congo red method of Bennhold (1922), which has a considerable degree of specificity, although it stains eosinophil granules, enterochromaffin granules and elastic tissue to some extent. The chief merit of this method (Fig. 81), details of which appear on p. 844, is that it affords a permanent preparation.

Dichroism. Structures which contain groupings which absorb light anisotropically exhibit the phenomenon of dichroism when viewed by polarized light. Cellulose fibres dyed with Congo red absorb green light very strongly if the plane of vibration of the light is parallel to the fibre axis, but not if the plane of vibration is perpendicular to this axis. Stated in another way, parallel orientated dye molecules will show different absorption coefficients towards plane polarized light, according to whether the plane of polarization is parallel to or at right angles to the axis of the molecules. In this case the inference is that the long dye molecules are lying along the fibre axis. This is shown below, diagrammatically, in Fig. 82.

The whole matter of the orientation of dyes on fibres is most ably

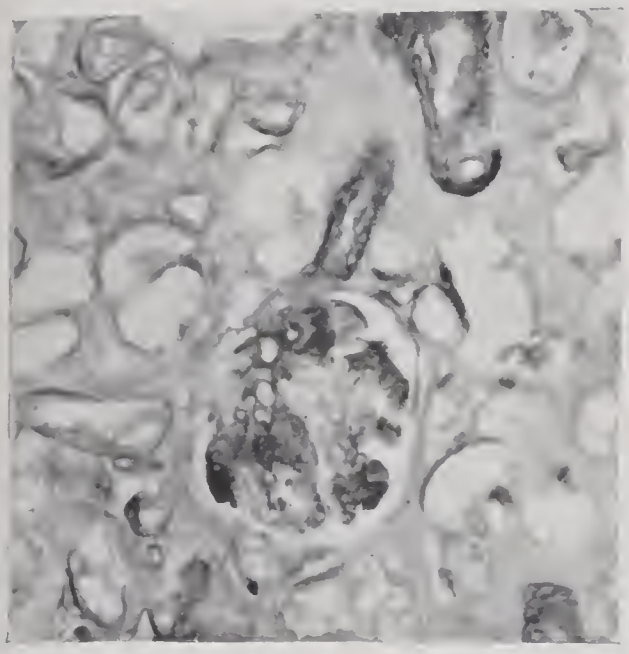

Fig. 80. Human kidney. Amyloidosis. Fresh frozen section. Shows strongly reacting deposits of amyloid in the glomerulus and elsewhere. Adams' DMAB —reaction. × 130.

Fig. 81. Human liver. Amyloidosis. Fresh frozen section. Amyloid deposits in the wall of a large sinusoid. Congo red—hæmalum. × 130.

Fig. 83. Human liver. As Fig. 81. Ferric hydroxamate reaction. × 130.

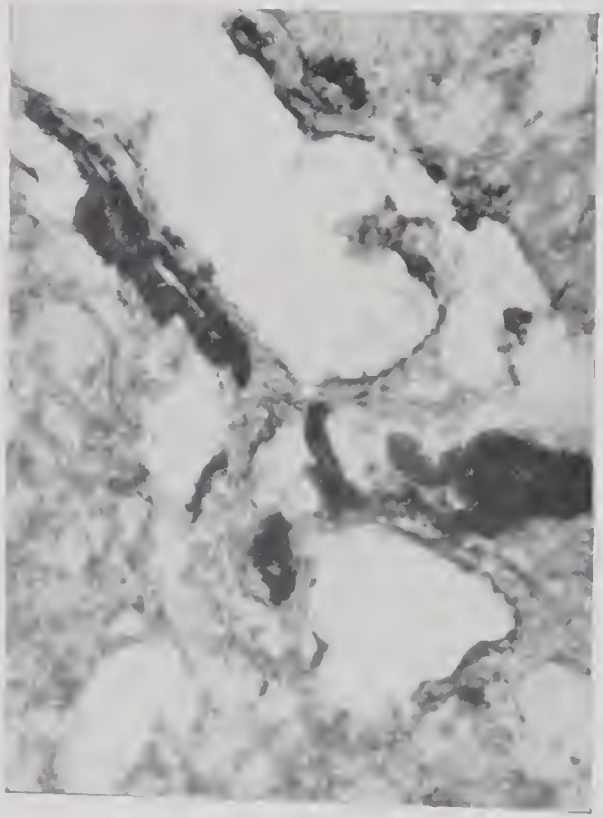

Fig. 84. Human liver. As Figs 81 and 83. Dialysed Iron reaction. × 510.

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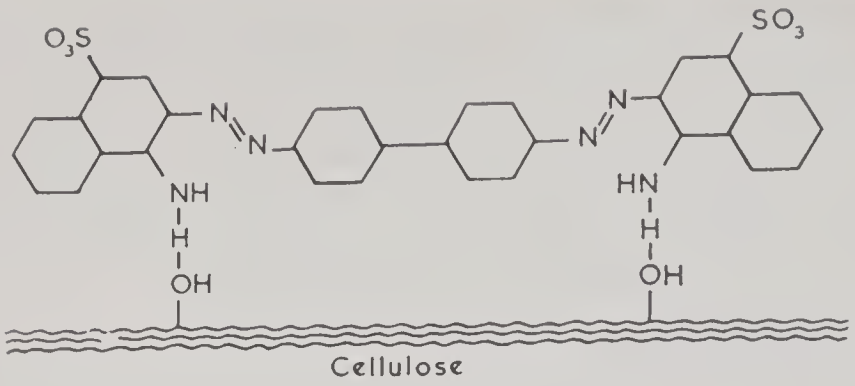

Fig. 82.

dealt with by Vickerstaff (1954), who points out that the dichroic effect is greatest at low dye concentrations, due possibly to preferential absorption on the most highly orientated parts of the fibre. Amyloid dyed with Congo red exhibits positive birefringence and dichroism and examination by polarized light should always be carried out if there is any doubt as to the identity of any Congo red-stained tissue component. Polarization optical studies on amyloid have been carried out by Pfeiffer (1953), Missmahl and Hartwig (1953) and Dieselben (1954). Many earlier studies on the same lines are referred to in the bibliography of these authors. More recently Diezel (1957) has reported on the dichroism of amyloid stained with Chlorantin fast red or with Evans blue. Seitelberger (1958) finds that amyloid stained by the coupled tetrazonium reaction gives anomalous colours when viewed by polarized light. He points out that Congo red and Evans blue are both diazo dyes and that the reaction product of the coupled tetrazonium reaction (see p. 90) is also a disazo dye. This effect may be regarded as characteristic, but not specific, for amyloid.

Enzymal Analysis

Amyloid is particularly resistant to attack by proteolytic enzymes. Studying this aspect of amyloidosis, Missmahl (1950) reported that samples of secondary amyloid would retain many of their staining properties after digestion with pepsin or after passage through the gastro-intestinal tract of the mouse. Stainability with Congo red and its attendant dichroism, for instance, withstood both these procedures, but methyl violet metachromasia (see below) was more labile. Wagner (1957) tested the effect on amyloid of incubation with trypsin, pepsin, pectin esterase, streptokinase, streptodornase and streptococcal and testicular hyaluronidase. Only very high concentrations of either pepsin or trypsin (10 mg./ml.) had any effect on protein staining or on mucopolysaccharide. High concentrations of testicular hyaluronidase decreased the staining of mucopolysaccharides only very slightly. Windrum and Kramer (1957) likewise observed

that partial peptic digestion of secondary amyloid deposits changed their staining properties from orthochromatic to metachromatic with toluidine blue or azure A. Subsequent digestion with testicular hyaluronidase removed most of the metachromatic material, suggesting that it is a polysaccharide of the chondroitin-sulphuric acid type. Resistance to enzyme attack is not usually used as a diagnostic criterion in the applied histochemistry of amyloid.

Tests for Polysaccharides

The positive PAS reaction of amyloid reflects the high carbohydrate content found in assays, but does not indicate how much of the staining is due to hexoses combined as mucoproteins and how much is due to complexing with mucopolysaccharide. Curiously enough, the alkaline tetrazolium reaction, which in the case of most mucins runs parallel to the PAS reaction, is almost invariably negative in the case of amyloid. The so-called paramyloid reacts readily, though it is, if anything, less PAS-positive. The ferric hydroxamic reaction (Chapter IX, p. 263) gives results which agree closely with those of the PAS reaction, provided the method is applied to unfixed sections. This is shown in Fig. 83.

The classical red metachromasia of amyloid with methyl violet is presumably due to the presence of acid mucopolysaccharides although, at least in fixed tissues, these are often difficult to demonstrate histochemically. An investigation into the nature of the dye-chromotrope bond in methyl violet-amyloid metachromasia was carried out by Carnes and Forker (1954, 1956). These authors found that methyl violet was bound metachromatically by amyloid at pH levels as low as 1·6, whereas the (non-metachromatic) binding of toluidine blue was abolished at pH 4·5. Differences were found in the spectral absorption curves for amyloid and cartilage (chondroitin sulphate). These and the above-mentioned dye-binding experiments suggested that amyloid possesses few strong anionic binding sites, and that methyl violet metachromasia depends on bonding of a different type from that associated with toluidine blue metachromasia of acid polysaccharides. In fresh cold microtome sections Hale's reaction (Chapter IX, p. 256) is strongly positive. This is illustrated in Fig. 84, p. 284. Staining with Alcian blue or Alcian green is also quite strong in such unfixed materials though in formalin-fixed frozen sections heating may be necessary before the dye can combine with the acid polysaccharide.

The methyl violet method (Appendix 10, p. 844) is usually regarded as the only specific stain for amyloid and the only means of distinction between true amyloid and other hyaline proteins which may resemble it superficially. The method suffers from impermanence ; fading

takes place with most samples of amyloid in a relatively short time, especially if paraffin sections are employed. With fresh sections, even if these are briefly fixed in alcoholic fixatives such as Wolman's acetic-ethanol, methyl violet metachromasia is more brilliant, more extensive and more permanent than it is in material from the same case prepared by conventional fixing and paraffin embedding. I consider that fresh, cold microtome, sections should be employed for all studies on amyloid, natural or experimental, particularly as there is in this case no prospect of loss by solubility in buffer or aqueous solutions.

Other Tests

Histochemical methods for lipids are usually negative in the case of amyloid, even when fresh sections are tested. This again seems to be unusual behaviour, since many extracellularly deposited proteins contain small amounts of lipid. These may be, as in the case of fibrin, included in the lattice of fibres during their formation. Nucleic acids are absent. Evidence from immuno-histochemical studies, already considered in Chapter VI, has confirmed the presence of γ-globulin in secondary amyloid deposits in human material. This constitutes a major advance in our knowledge of the nature of amyloid. We are still mainly ignorant, however, of the mechanism responsible for the deposition of this globulin and its combination with acid muco-polysaccharide to form the insoluble, enzyme-resistant complex which we call amyloid.

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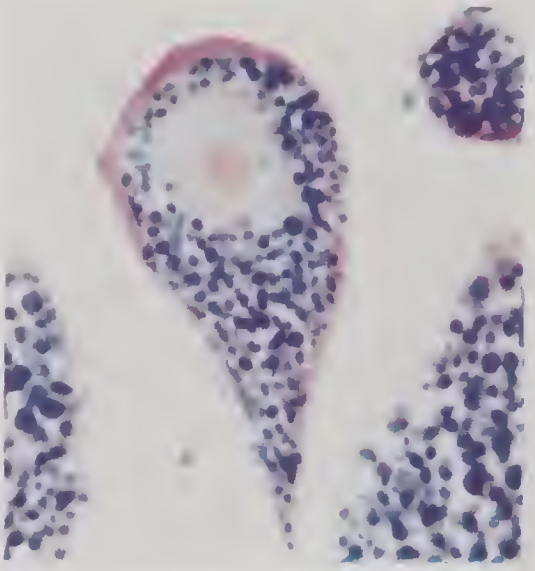
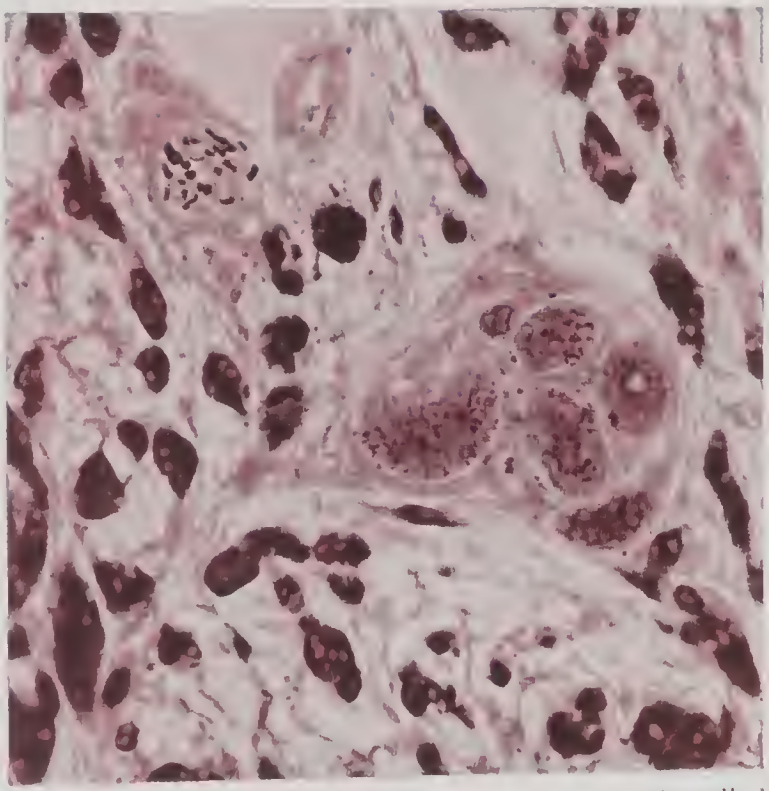
III A. Human spinal cord. Anterior horn. Frozen section from a case of gargoylism. Shows positive reaction of stored material within the cells. Dialyzed iron method. × 150.



III B. *Buccinum undatum* (whelk). Oocyte. Development of phospholipid granules in an early stage. Copper phthalocyanin—Neutral red. × 400.



III C. Human skin (10 μ frozen section). So-called granular myoblastoma. Myelin sheaths, fat globules and the tumour cell granules stain strongly. Sudan black B, carmalum. × 150.

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CHAPTER XI

LIPIDS, LIPOPROTEINS AND PROTEOLIPIDS

Before presenting any classification of lipids as a basis for the discussion of their histochemistry, it is necessary first to deal with the nomenclature to be employed. In the case of no other class of substances is there such wide variation in the significance attached to the various descriptive terms. First of all we have to consider the words lipoid, lipid, lipine and lipin.

Cain (1950), in his review of the histochemistry of the substances we are considering, used the word lipoid in the sense in which it was used by Baker (1946a) to mean "all those substances that can be extracted from tissues by several or all the usual fat solvents, and are insoluble or only colloidally soluble in water." The term "lipine" he restricted to substances containing N as well as C, H and O, such as lecithin, kerasin, and allied compounds. In this chapter, instead of the word "lipoid," the word "lipid" is used, and this covers the whole of the large group of naturally occurring fat-like substances which are insoluble in water but soluble in such solvents as benzene, chloroform, acetone, ether, etc. The word "lipoid," if it is used at all, and the word "lipin," are used to refer to the substances classed as "lipines" by Cain. Since the ending "ine" has a definite chemical significance, it has been thought better to avoid use of the word lipine altogether.

Much of the lipid present in mammalian tissues is associated with protein. The so-called masked lipids of the histologist belong to this category although in some cases the phenomenon of unmasking may be due to variation in the degree of dispersion of lipid droplets. Lipoproteins can be defined as substances which behave as proteins, from which lipids can be extracted and proteolipids (Rossiter, 1955) are substances which behave as lipids, from which proteins can be obtained.

Classification of Lipids

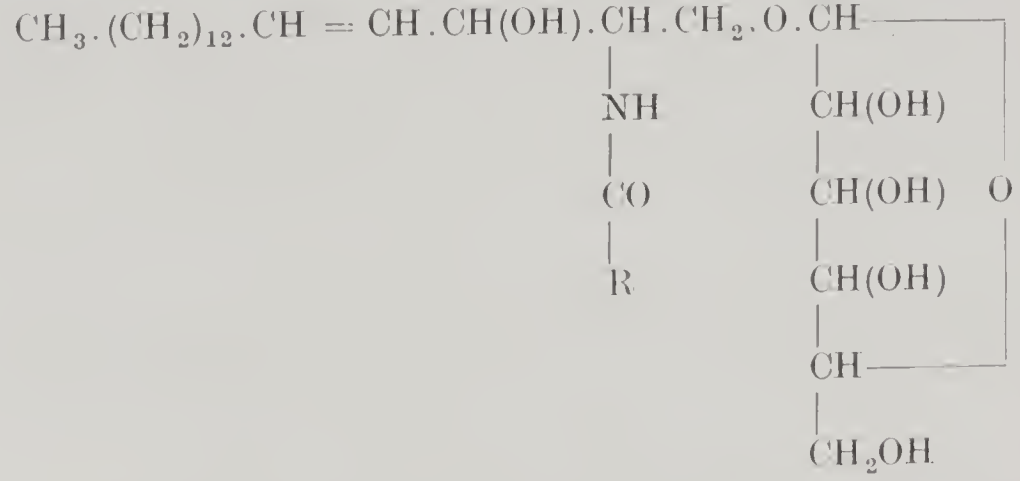
According to Lovern (1955) there is no field of biochemistry in which there is less uniformity than the lipids. This is not a promising start for the histochemist, who is bound to adhere to some classification if his work is to be of any significance in the wider sense of the term. The basic classification used below is derived from Bloor (1943).

obtained from the sphingomyelins on hydrolysis and these differ from those found in the glycerophosphatides chiefly in being much more saturated. The chief acid is the saturated C_{24} derivative lignoceric acid.

Folch and Sperry (1948) have proposed that the terms lecithin, kephalin and sphingomyelin be used only for the crude fractions and that the three groups of compound ester phosphatides be designated : (1) phosphoglyeerides (lecithin and kephalin), (2) phosphoinositides (lipid containing phosphoric acid and inositol such as the diphosphoinositide of brain), and (3) phosphosphingosides (sphingomyelin). The disadvantage of this classification is that the acetal phosphatides cannot be fitted into it.

The acetal phosphatides, which are always found mixed with the ester phosphatides in varying quantities (up to 12 per cent.), are similar to them in composition but yield higher aldehydes (stearic or palmitic aldehydes) on hydrolysis, in place of fatty acids. Because their histochemistry is closely connected with that of aldehydes in general the acetal phosphatides are considered, together with other substances containing these groups, in the following chapter.

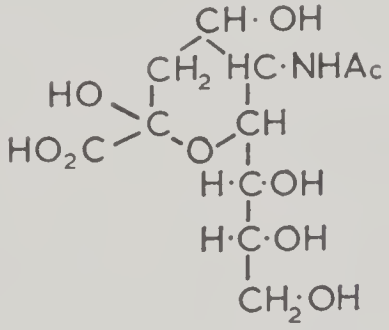
Glycolipids. The cerebrosides contain fatty acids, a carbohydrate (usually glucose or galactose) and a complex alcohol such as sphingosine ($CH_3.(CH_2)_{12}.CH = CH.CHNH_2.CHOH.CH_2OH$), but no phosphoric acid. The sugar is usually D-galactose but sometimes it is D-glucose and the fatty acid may be unsubstituted or it may contain a hydroxyl group in the α-position. In the latter case it may be called a phrenosin whereas the former type is known as a kerasin. The cerebrosides have the following general formula :



It is apparent that they bear a close resemblance to sphingomyelin.

The gangliosides (Klenk, 1942) are cerebroside-like substances which are found chiefly in the ganglion cells of the nervous system. They yield on hydrolysis 1 mole each of fatty acid, sphingosine and neuraminic acid, and 3 moles of galactose. Small proportions of

glucose accompany the latter. The characteristic component of the gangliosides is the hypothetical amino-sugar neuraminic acid, whose derivatives are found in many mammalian tissues in combined form. The naturally occurring derivatives are known as sialic acids (Blix, Gottschalk and Klenk, 1957). The commonest substituent is acetyl and Gottschalk (1955) proposed the formula given below for *N*-acetylneuraminic acid. It may be regarded as the product of aldol condensation between an *N*-acetylhexosamine and pyruvic acid.



N-acetylneuraminic acid

The gangliosides are localized chiefly in the grey matter of the C.N.S., as mentioned above, although Brante (1951, 1952) has found small quantities in white matter.

Cerebrosides, gangliosides, sphingomyelins and " kephalin B " were included by Carter *et al.* (1947) under the common heading of sphingo-

TABLE 18

Nomenclature of Lipids

Sulphatides	= sulpholipids = sulphur-containing lipids.
Phosphatides	= phospholipids = phosphorus-containing lipids.
Glycerol phospholipids	= lecithins and kephalins = mono-amino-phospholipids = phosphoglycerides and phosphoinositides (inositol-containing kephalins) = KOH-hydrolysable phospholipids.
Sphingolipids	= all sphingosine-containing lipids.
Phospho-sphingosides	= all phosphorus-containing sphingolipids = sphingomyelins and " kephalin B " = KOH-resistant phospholipids.
Sphingomyelins	= diamino-phospholipids.
Glyco-sphingosides	= all hexose-eontaining sphingolipids = cerebrosides and gangliosides = glycolipids (Glycolipids often called galactolipids since nearly all the hexose in *normal* tissues is galactose).

lipids. This is a useful sub-classification to the main one used here which emphasizes the common factor (sphingosine) present in all the above-mentioned lipids. The first two are glycosphingosides and the last two phosphosphingosides. "Kephalin B" is the name given by Brante (1949) to a sphingomyelin-like compound which, like sphingomyelin itself, is resistant to mild hydrolysis with N-KOH.

The table of synonyms given on p. 293 is derived from Edgar (1956).

Derived Lipids

Under the heading of derived lipids are classified, *inter alia*, the various fatty acids which can be derived from simple and compound lipids by hydrolysis. These fatty acids are of two types, unsaturated or saturated, depending on the presence or absence of double bonds in their molecule. Stearic and palmitic are the chief saturated fatty acids and oleic acid the chief unsaturated fatty acid occurring in animal fats. The sterols (cholesterol) are usually placed in the class of derived lipids. In this chapter we have to consider histochemical methods for distinguishing cholesterol and its esters, neutral fats, fatty acids, phosphatides and cerebrosides. In the following chapter we shall consider methods for the ketosteroids and acetal phosphatides.

Cain (1950), in his review of the subject, emphasized the fact that lipids are really less difficult to analyse histochemically than carbohydrates or proteins. He suggested that the lack of histochemical studies on lipids was due to a general mistrust of the techniques, and his independent studies of the latter have gone some way to remedy the situation. A feature of lipid histochemistry is its comparative freedom from problems of location. Many lipids occur in droplets, sharply separated from the surrounding aqueous cytoplasm. That this appearance is not fixation artifact is shown by their demonstration in living cells as well as in freeze-dried material. According to Cain the histochemist must be prepared to recognize four possible groups of lipids :

(1) Lipids detectable as such in living and fixed tissues.
(2) Lipids detectable as such in fixed tissues only (but present in living tissues).
(3) Lipids detectable as such in fixed tissues only (not present in living tissues).
(4) Lipids detectable only after special processes additional to fixation.

The methods for detecting and analysing lipids are divisible broadly into two large sections, physical and chemical.

Physical Methods for Lipids

Solubility in Organic Solvents (Extractive Methods)

An important characteristic of the lipids is their ability to dissolve in each other. The rules of solubility are good only for pure substances and pure solvents. Lison (1936) pointed out the difficulties attached to the analysis of lipids by reference to their solubilities, maintaining that the prolonged extraction techniques of the chemists could not be imitated by histochemical procedures. These strictures, which Lison (1953) still maintains, must be borne in mind when applying the methods described below.

The performance of the various tests for lipids after the application of known lipid solvents and comparison with unextracted materials, has long been customary. Four extractive techniques must be mentioned as a preliminary stage to the consideration of extractive techniques as a whole, in relation to lipid histochemistry.

Ciaccio's Method II. This method, which was evolved by Ciaccio (1934), must be distinguished from his method I for phospholipids, referred to later in this chapter. It consists essentially of acetone extraction of formalin-fixed blocks followed by staining either with Sudan III or with the Smith-Dietrich procedure. Previous authors had found that simple acetone extraction of blocks, followed by staining for lipids with Sudan III, gave unsatisfactory results. Ciaccio added the refinement of short extraction in ascending strengths of acetone (70, 80 and 90 per cent.) containing 1 per cent. cadmium nitrate, followed by prolonged extraction in acetone containing 2 per cent. of a saturated solution of cadmium nitrate in absolute alcohol. Passing thence to 50 per cent. acetone in water by numerous stages, all in the presence of cadmium, Ciaccio postchromed his material for 2–3 days in acetone-dichromate, embedded in paraffin and stained with Sudan III. Lison (1936) noted that better results were obtained by means of Sudan black B but remarked (1953) that although the method was logical it had been found unsatisfactory in the hands of a number of workers. I agree with Lison that the main fault in Ciaccio's method lies in the fact that when present in mixtures phospho- and glycolipids are to a considerable extent soluble in acetone and acetone-cadmium mixtures. Ciaccio (1956) introduced modifications of his original method designed to increase its specificity. I have not tested these, however.

Baker's Pyridine Extraction Method. In order to distinguish between lipin and non-lipin substances staining by his acid hæmatein method, Baker (1946b) employed an extraction technique using pyridine at 60° on material fixed in weak Bouin. The extraction stage was followed by postchroming and by the full acid hæmatein routine.

The special weak Bonin fixative was necessary in order to permit total extraction of phosphatide by the pyridine. This technique can be criticized for employing a different fixative to that used for the test sections which are not subjected to extraction. This does not necessarily invalidate the interpretation put upon it by the original author. There are, however, a number of recent observations which make exact interpretation difficult. These are considered below.

Keilig Extraction Methods. Working on unfixed blocks of human brain, Keilig (1944) used the regular series of extractions given below. Blocks of tissue not more than 3 mm. thick were treated with the solvents shown in the first column with the results indicated in the second.

(1) Cold acetone . . .	Removes glycerides, cholesterol, cholesterides and ketosteroids.
(2) Hot acetone . . .	Removes cerebrosides.
(3) Hot ether . . .	Removes lecithins and kephalins.
(4) Hot chloroform/methanol .	Removes all lipids.

Each extractive stage was performed over a period of 24 hours with at least three changes of the solvent. All but the first should be performed in some kind of refluxing apparatus. Keilig herself used a Soxhlet apparatus for her studies on brain ; I used a simple apparatus consisting of a reflux condenser over a flask heated by an electric light bulb in a metal housing. The correct temperature was obtained by altering the distance between bulb and flask. Lhotka (1955) modified the standard Soxhlet apparatus by adding a water-jacket around the extraction chamber so as to keep the temperature of tissues undergoing extraction below limits considered damaging. After extraction the blocks of tissue are carried rapidly through descending strengths of alcohol to water and frozen sections are cut and stained with Sudan black B. If any of the Sudan dyes are used for final staining it is essential that no formalin and no mordant of any kind be used after extraction. In practice the results are not usually as clear cut as suggested in the above list, although removal of the various substances occurs as stated. With the phosphatides, for instance, although extraction by hot acetone fails to remove them, a variable dispersive effect is noted so that localization is impossible and the general effect is dirty. This is not a serious drawback since accurate localization will have been made by other methods. As an alternative to this procedure, if phosphatides are the object of study, the extracted blocks may be postfixed in formalin, postchromed, and frozen sections subjected to a Smith-Dietrich type of staining (see p. 311).

Okamoto-Ueda Extraction Methods. In order to distinguish between the various lipids stained by their mercuric nitrate-diphenyl-

carbazone method Okamoto and his associates (Ueda, 1952) performed a series of extractions on tissue sections *after* the staining procedure. The latter, which is fully considered on p. 314, was presumed to stain all phospholipids and glycosphingosides bluish-purple. After 48 hours' treatment with pyridine at 0–4° glycosphingosides were said to be removed. Subsequent treatment with ether at 22° for 48 hours was considered to extract, in addition, all phospholipids except sphingomyelins. These methods were employed by Diezel (1954) in his studies on the primary lipidoses, in a slightly different manner. He performed the extractions first and the staining afterwards, without suggesting any different interpretation.

Extraction Techniques in General. With all extraction techniques using organic solvents it is necessary to be aware of deviations from the accepted rules of solubility derived from biochemical estimations. Edgar and Donker (1957) have performed a valuable service in checking by chemical methods the amount of the various lipid fractions extracted from tissue sections by procedures of the Ueda type. They found very considerable differences between fresh and formalin-fixed sections. From the latter 80–90 per cent. of the total glycosphingoside was removed by 48 hours' treatment with cold pyridine. The percentage of ganglioside resistant to removal by pyridine was observed to be greater in cases of amaurotic idiocy than in diffuse sclerosis, in both of which there is an accumulation of these substances in the brain. Pyridine treatment also dissolved some of the KOH-hydrolysable and some of the KOH-resistant phospholipids from formalin-fixed sections. Brante (1949), however, showed that formalin causes part of the original KOH-hydrolysable phospholipid to disappear and that it converts another part of this fraction into a KOH-resistant lipid. This means that in formalin-fixed tissues this last fraction consists not only of phosphosphingosides but is a mixture of lipids of unknown constitution.

Edgar and Donker observed that treatment with ether for 48 hours at room temperature had no effect whatsoever on the glycosphingoside content of the sections (as expected). Moreover, except in cases of Niemann-Pick's disease it had no material effect on the phosphosphingosides. In fresh material cold pyridine extraction did not decrease the phosphosphingoside content of sections, and surprisingly, it failed to dissolve the KOH-hydrolysable phospholipids although lecithins and kephalins are supposed to be soluble in pyridine. In agreement with theoretical expectations much of the KOH-hydrolysable phospholipid was dissolved by treatment with ether but a considerable quantity (10–40 per cent.) remained insoluble. On the other hand, Edgar and Donker found that a considerable part of the KOH-resistant phospholipid, which is theoretically insoluble in ether,

was dissolved by this treatment. They considered that this anomaly might be due to Brante's "kephalin B" which is soluble, like other kephalins, in wet ether. If this is so, the ether-soluble part of the KOH-resistant fraction in fresh tissues can be identified with "kephalin B." Edgar and Donker concluded from their results (1) that Okamoto-Ueda extractions are unsuitable for application to fresh tissues. (2) That in the case of formalin-fixed tissues a material *totally* unaffected by cold pyridine is unlikely to be a glycosphingoside. (If a material is partly dissolved, however, the residual part cannot be identified as phospholipid since it may belong to the insoluble fraction of the glycosphingosides.) (3) That ether extraction cannot be assumed to distinguish between phospholipid and sphingomyelins. Table 19, below, gives a list of Edgar and Donker's observations with the theoretical results side by side for comparison.

TABLE 19

Theoretical and Observed Solubilities of Tissue Section Lipids

Lipid Fraction	Pyridine			Ether		
	Expected	Fixed	Fresh	Expected	Fixed	Fresh
Cerebrosides	Sol.	10–15% Insol.	70% Insol.	Insol.	Insol.	20–30% Sol.
Glycosphingosides						
Gangliosides	Sol.	10–15% Insol.	70% Insol.	Insol.	Insol.	20–30% Sol.
Sphingomyelins	Insol.	20–30% Sol.	Insol.	Insol.	Insol.	50–70% Sol.
KOH-resistant phospholipids						
"Kephalin B"	Not known	20–30%	Insol.	Insol.	Insol.	50–70% Sol.
Kephalins	Sol.	20–70% Insol.	Insol.	Sol.	Insol.	20–50% Insol.
KOH-hydrolysable phospholipids	Sol.					
Lecithins	Sol.	20–70% Insol.	Insol.	Sol.	Insol.	20–50% Insol.

In the case of Baker's hot pyridine extract the results on formalin-fixed (or weak Bouin-fixed) tissues may also be confusing. Some work carried out by Almeida and Pearse (1958) on the development of myelin in rabbit brain illustrates this point. Up to the 11th post-natal day all acid hæmatein-positive lipids were rendered negative by hot pyridine. After this day, however, there was an increasing amount of residual staining in the myelin sheaths which could be demonstrated also by means of Sudan black B staining and by copper phthalocyanin but not by the Feyrter method. There was no reason to doubt the lipid nature of the residual material since it was extractable with chloroform-methanol solutions. If Baker is correct in his assumption that acid hæmatein does not stain cerebrosides the residual material might be regarded as phosphosphingoside. This substance, however, does not stain with alcoholic solutions of copper phthalocyanin and we found, in *in vitro* tests, that a commercial sample of cerebroside was strongly stained with acid hæmatein. Cerebrosides have been

shown by many authors to be strongly positive by the very similar Smith-Dietrich procedure (Edgar, 1956 ; Lennert and Weitzel, 1952 ; Landing and Freiman, 1957). In any case, extraction with hot pyridine has been shown to be partially ineffective.

The scepticism expressed by Lison and by Edgar (1956) and by myself, with regard to the use of lipid extraction techniques in histochemistry is still to be upheld in the light of modern experience. This does not mean, however, that such techniques should not be attempted though wherever possible they should be controlled by studies of the type carried out by Edgar and Donker. I believe that a series of extractions should be devised for application in the first instance to unfixed cold microtome sections. These could be secondarily post-fixed in formalin, dichromate or chromic acid as an additional means of differentiation. With the cold microtome the handling of alternate sections for histochemical treatment and for chemical assay is a matter of great ease and convenience.

Certainly in the early stages of the development of " certified " extractions for histochemical use it will be necessary to follow the normal practices of lipid chemistry but *without* adopting their conclusions. An excellent survey of extraction techniques is given by Lovern (1955) in the second chapter of his book on the " Chemistry of the Lipids." In view of its well-known effect in liberating protein-bound lipids ethanol and ethanol mixtures will probably have to be avoided as fixatives for fresh tissue sections destined for lipid studies. Since cold acetone is a poor solvent for phosphatides and sphingolipids it may be useful as a substitute, and in order to depress their solubility still further electrolytes such as magnesium chloride may be added to the acetone. Formalin and formol-calcium mixtures may also be used but even with thin sections fixation with this and the various dichromate mixtures is likely to be slow. As Lovern (1955) remarks there is an immense volume of published material on lipid extraction techniques and many possible variations of solvent mixtures and sequences. Clearly only the fringe of the problem has been approached by histochemists and some resolute and painstaking studies combining histochemical and chemical methods in alternate serial cold microtome sections are absolutely necessary.

Examination by Polarized Light

Very little assistance can be obtained, in distinguishing between neutral fats, fatty acids, phospholipids and glycolipids, from examination by polarized light. Lison (1936, p. 198) put the position very clearly. Neutral fats, examined in the fresh state, may be liquid and therefore monorefringent, but on cooling they may set in the form of birefringent crystals. Fatty acids in the crystalline state are also

birefringent, but may be present in tissue sections in an amorphous isotropic state. It seems, therefore, that in the fresh state both neutral fats and fatty acids are monorefringent but that when cooled, or when fixed in formalin, both may become crystalline. The cholesterol esters and lipins behave in an identical manner. Between certain ranges of temperature they exist as liquid spherocrystals which give Maltese cross birefringence. At temperatures above their melting points they are isotropic and below a certain point they may exist as acicular crystals giving only plain refringence. Lison summed up by stating that examination by polarized light alone gave very ambiguous information regarding the chemical nature of the fats. Three conditions may be found, according to this author (1953, p. 366).

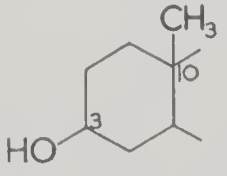
(1) *Lipids Observed to be Monorefringent.* No conclusion is possible. Glycerides and fatty acids in the melted state are never birefringent. Cholesterol esters and lipines can be but are not always so, if conditions are such as to prevent the formation of liquid crystals.

(2) *Lipids Birefringent without Maltese Cross.* In this case they behave as true crystals, in the solid state. When the stage of the microscope is rotated through 360° there are four positions of extinction. No diagnostic conclusion is possible since all lipids can exist in this condition.

(3) *Lipids show Maltese Cross Birefringence* (Fig. 85). The only conclusion which can be made is that they consist either of cholesterol esters or of lipines. Glycerides and fatty acids never exist in the form of spherocrystals.

In the case of conditions 1 and 2 an attempt can be made to turn the lipid into a liquid crystalline state. This is carried out in the first case by chilling or by mounting in levulose syrup and in the second case by warming. If spherocrystals appear this confirms the presence of cholesterol esters or lipines but their absence cannot be presumed in the case of failure to observe spherocrystals.

Digitonin Reactions. Windaus (1910) showed that free cholesterol (but not its esters) treated with alcoholic solutions of digitonin, yielded a crystalline complex insoluble in water, acetone and ether but soluble in glacial acetic acid or pyridine. Digitonin precipitates in general those steroids which have a 3 β-hydroxyl group as shown here, the



hydroxyl group at C(3) being on the same side of the ring system as the methyl group at C(10). The reaction was first used in histochemistry by Brunswick (1922). He used a 0·5 per cent. solution of digitonin in

85 per cent. alcohol and examined the sections, in this medium under a coverslip, for the birefringent crystals of digitonin-cholesterol. Leulier and Revol (1930) used digitonin in 35 per cent. alcohol and distinguished the birefringence of digitonin-cholesterol from that of the cholesterol esters by subsequently staining the sections with a red Sudan dye. The esters became coloured and lost their birefringence while the crystals of the digitonin complex remained anisotropic and colourless. Bennett (1939) also employed the reaction in his studies on the adrenal cortex of the cat. Though the digitonin reaction is specific for free sterols, it is of little use for showing the presence of ketosteroids in the adrenal cortex since the birefringence due to these (if any) cannot be distinguished from that of the accompanying cholesterol esters, even by the Leulier-Revol technique.

In practice useful results are seldom obtained by this technique. In particular it is usual to find that all the lipids in a digitonin-treated section stain with the Sudan dyes and with Oil Red O and that digitonin cholesterides are not to be distinguished. Since the latter are supposedly insoluble in cold acetone, and since free cholesterol and cholesterol esters are freely soluble, a further basis for differentiation is offered. Sometimes one can observe acetone-insoluble crystals after digitonin treatment of fresh tissue sections but the doubts expressed in the previous section of this chapter do not allow the clear conclusion that these represent cholesterol digitonides.

Primary Fluorescence Methods

Examination of lipids by means of the ultraviolet microscope is of little importance in histochemistry, except in the case of the oxidation products of lipins which are better known as lipofuscins. These are fully considered in Chapter XXIII. If carotenoid pigments are dissolved in lipids they may impart to them a strong fluorescence, but no information as to the nature of the solvent lipid can be obtained therefrom.

Secondary Fluorescence Methods

These methods depend on the solution of fluorescent substances, usually referred to as fluorochromes, in the lipids of the tissues. A number of dyes have been used for the purpose, including methylene blue, Thioflavin S, Rhodamine B, Rose Bengal, Magdala red and Phosphine 3R. This last dye was considered by Volk and Popper (1944) to reveal the presence of lipids other than fatty acids, soaps and cholesterol. Its great advantage over the usual staining methods for lipids lies in the use of aqueous solutions so that the smaller fat droplets are not dissolved out during staining. Phosphine 3R imparts a clear silvery-white fluorescence to lipids in which it is dissolved and this is easily distinguished from the natural fluorescence of the tissues

without employing control sections. Details of this method are given in Appendix 11.

A second method for the demonstration of lipids, depending on the development of secondary fluorescence in ultraviolet light, is the benzpyrene-caffeine method of Berg (1951). This method has the same kind of advantages as the Phosphine 3R technique and it is particularly useful as a sensitive method for demonstrating lipids in fine structures, occurring as lipoprotein complexes, or in fine disperse form. It is not suitable as a routine technique but only for application to problems of research into the finer distribution of lipids. Benzpyrene is apparently soluble in all lipid substances, including cholesterol and its esters, so that it cannot be used as the basis of a method for distinguishing the various types of lipid. Berg's method is given in Appendix 11, and the results of its application to mast-cell granules are illustrated in Fig. 86. For comparison Fig. 87 shows them stained by colloidal Sudan III.

Fat-soluble Colorant Methods for Lipids

Historical Survey

The most widely used techniques for lipids depend on the solubility of the inert bis-azo, or other, dyes in the fats themselves and for this purpose the red dyes Sudan III and Sudan IV are commonly employed. The more strongly red Sudan IV has largely replaced Sudan III as the most important single fat stain, though more recently a number of dyes, in related and unrelated groups, have been tried out. Some of these are mentioned below and in Table 20, which is a historical survey of the use of colorant dyes and other methods for staining fats.

The first group in the Table comprises the classical as well as most of the commonly employed fat-staining techniques. The third group also contains methods in common usage. The second and fourth groups are really the same, since both make use of the principle of colloidal suspension of the " dyes " employed. The fifth group is the only one using a relatively new principle, the solution of the " dyes " in hydrotropic detergents, while the sixth and last group contains the newer fluorescent techniques as well as Lorrain Smith's classical method for fatty acids.

Various Solvents for the Red Sudan Dyes

Variations in the use of the Sudan and other dyes depend largely on the type and concentration of the fluid in which they are dissolved or suspended, but in each case the solubility of the dye in fat must exceed its solubility in the solvent. Herxheimer's (1903) mixture (equal parts acetone and 70 per cent. alcohol) remains a popular solvent

TABLE 20

Historical Survey of Fat-staining Methods
(After Berg, 1951)

Colorant	Solvent	Remarks	Author
		Simple solutions	
Sudan III	Alcohol 70 per cent.	Adipose tissue, fat droplets	Daddi (1896)
Scharlach R and Sudan IV	Alcohol 35 per cent. Acetone 50 per cent.	Adipose tissue, fat droplets	Michaelis (1901) Herxheimer (1901)
Sudan Brown	Isopropanol	Fine fat droplets, myelin sheaths ; rapid intensive staining	Lillie (1944)
Oil Red 4B	Pyridine 70 per cent.		Proescher (1927)
Oil Red O			
Scharlach R	Diacctin 50 per cent.	Adipose tissue, fat droplets	Gross (1930)
Sudan red	Diacetin 50 per cent.	Adipose tissue, fat droplets	Domagk (1933)
Sudan black B.	Alcohol 70 per cent.	Mitochondria. Leucocyte granules. Bacterial lipids	Lison and Dagnelic (1930) Sheehan (1939) Hartman (1940) Leach (1938)
Sudan black B.	Diacetin 50 per cent.	Myelin, etc.	
Sudans	Propylene glycol	Mitochondria. Finest granules	Chiffelle and Putt (1951)
		Colloidal solutions	
Sudan III	Alcohol 40 per cent.	Leucocyte granules, diffuse cytoplasmic stain	Romeis (1927)
Sudau III	Alcohol-phenol-acetic acid	Leucocyte granules, " Sudanophobic " lipids	Jackson (1944)
Sudan III	Acetic acid-gelatine	Leucocyte granules, " Sudanophobic " lipids	Govan (1944)
		" Oversaturated " solutions	
Sudan III and IV	Alcohol 70 per cent.	Improved staining of lipids	Kay and Whitehead (1935) Kay and Whitehead (1941)
Sudan IV	Alcohol 65 per cent.	Improved staining of lipids	Froeboese and Spröhnle (1928)
		Serum solutions	
Sudan III	Serum	Vital staining of lipochondria	Ludford (1934)
3 : 4-benzpyrene	Glycerine and serum	Vital staining. Bacteria	Günther (1941) Graffi (1939)
3 : 4-benzpyrene	Glycerine	Free fat and structure lipids	Graffi and Maas (1938)
		Hydrotropic solutions	
Sudan IV or Scharlach R	Trichloroacetic acid Sulphosalicylic acid Caffeine solutions	Finest fat droplets. Diffuse cytoplasmic staining. Myelin sheaths	Hadjioloff (1938)
3 : 4-benzpyrene	Caffeine solution	Critical staining of small amounts of lipid and lipoproteins	Berg (1951)
		Aqueous solutions	
Nile blue	Water	Unsaturated fatty acids pink. Lipids blue	Smith (1907) Smith and Mair (1911)
Rhodamine B	Water	Vital staining of neutral fats (plant)	Strugger (1937, 1938)
Phosphine 3R	Water	Lipids except cholesterol, fatty acids and soaps	Popper (1944) Volk and Popper (1944)

for the red Sudan dyes, although there are strong objections to its use on account of the ready solubility of small fat droplets in this solution and on account of the tendency of the dye to crystallize out of solution during differentiation. Dissatisfaction with the results of staining by means of the red Sudan dyes dissolved in concentrated alcoholic solutions has led to the employment of weaker alcoholic solutions for longer periods in an effort to avoid extraction of the smallest fat globules. Kaufmann and Lehmann (1926) made a series of experiments with lipid substances incorporated in elder pith in which, for staining glycerides and lipins, they used a 40 per cent. alcoholic solution of Sudan III. Romeis (1927) used a similar solution for staining fats in frozen sections. Froeboese and Spröhnle (1928)

used the Romeis method and concluded that the superior staining of fat which was obtainable, though irregularly, with 40 per cent. alcoholic solutions was due not to the preservation of small lipid droplets but to the colloidal properties conferred on the stain by the increased water content. Kaufmann and Lehmann (1929) conclusively demonstrated by chemical estimation that 70 per cent. alcohol dissolves fatty substances from frozen sections at a considerable rate and Kay and Whitehead (1934) considered that this indicated that the chief merit of the Romeis technique lay in the superior fat-preserving properties of 40 per cent. alcohol. These authors (1935), having previously found Sudan III to be unsatisfactory, used saturated Sudan IV in absolute alcohol, diluted to 66 per cent. with water before use. Romeis (1929) brought out a modification of his original technique using weak alcoholic solutions of a fraction of commercial Sudan III which he called Sudan orange. Technical difficulties in the preparation of his solution and the fact that other authors could not duplicate his results, prevented the general adoption of the method.

Colloidal Suspensions

Jackson (1944) evolved a new method of Sudan staining as the result of attempts to demonstrate histochemically in the cells of a contagious venereal tumour of dogs, a lipid substance which was resistant to staining by ordinary Sudan methods but which was partly revealed by the Romeis technique. His staining solution consisted of a colloidal suspension of Sudan III produced by adding 5 per cent. aqueous phenol to an 80 per cent. alcoholic stock solution of the dye until the alcohol content was reduced to 60 per cent. After standing for a few hours, glacial acetic acid was added in the proportion of 2·5 drops per ml. of carbol Sudan, the mixture being filtered and allowed to stand 24 hours before use. Using frozen sections, Jackson stained these in his acetic-carbol-Sudan for $1\frac{1}{2}$ hours or more and differentiated in acetic acid-alcohol. Besides neutral fats, with this technique other lipids, some of which were probably phosphatides, stained bright orange. Some weak staining of phosphatides occurs when the Sudan dyes are used even in the more conventional techniques and, since many superior methods for phosphatides now exist there is no point in using either the Romeis or Jackson methods for their appreciation.

Work on different solvents for the Sudan dyes continued and Govan (1944), following the method of Hadjioloff (1938), went further than previous authors in avoiding the use of fat solvents altogether. He used a colloidal suspension of either Sudan III, Sudan IV, or Sudan black B in 1 per cent. aqueous gelatin containing 1 per cent. acetic acid, staining frozen sections for 30 minutes at 37°. With this

FIG. 85. Myelin sheaths photographed by polarized light to show "maltese-cross" birefringence. × 350.

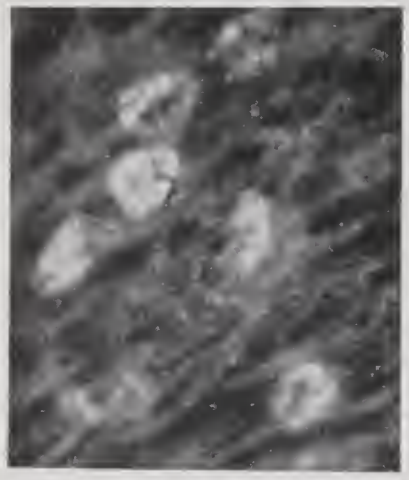


FIG. 86. Mouse myometrium. Mast cell granules showing strong fluorescence in ultraviolet light. Benzpyrene method (Berg). × 540.

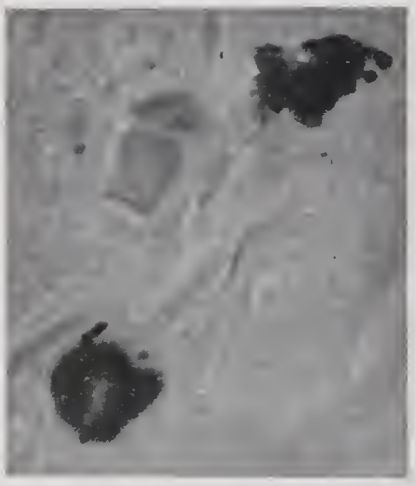


FIG. 87. Human stomach. Mast cell granules stained for lipid. Colloidal Sudan IV. × 1000.

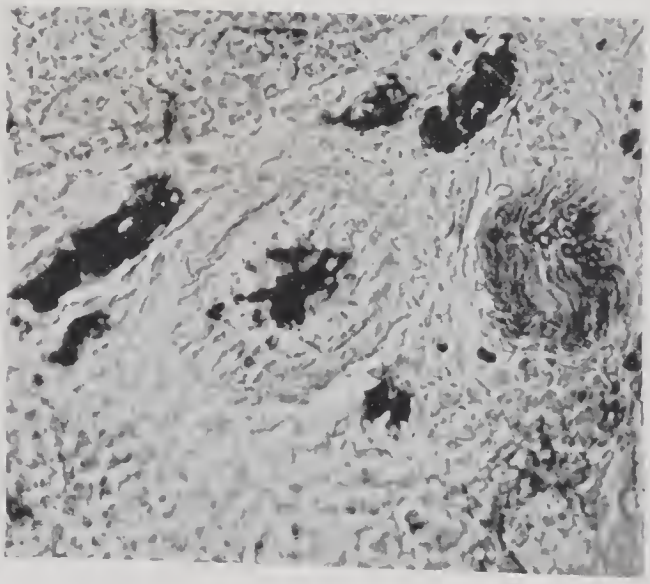


FIG. 88. Frozen section (10 μ) of rat lung. Red cells appear black, myelin sheaths (below) dark blue. Acid hæmatein method (Baker). × 235.

[*To face p.* 304.

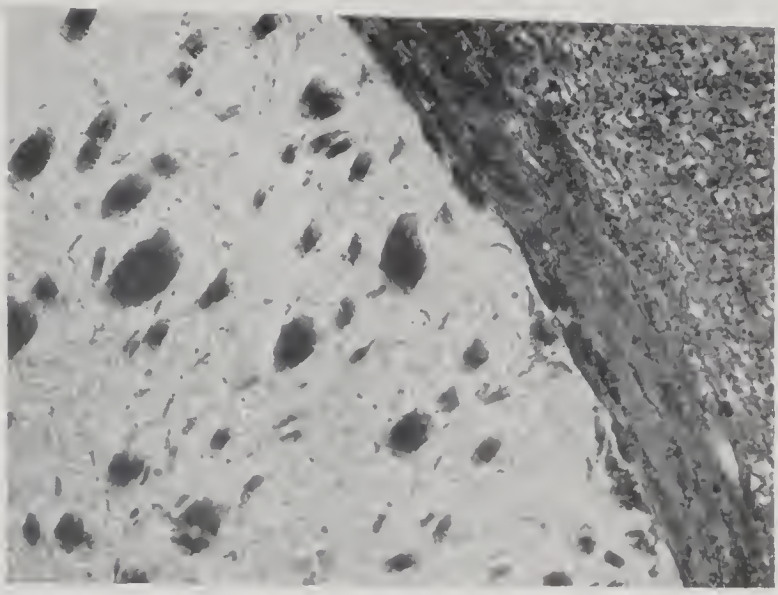


FIG. 89. Rabbit brain. Shows purple staining of myelinated tracts. Okamoto method. × 36.

FIG. 90. Rabbit brain. Bright blue staining of myelinated tracts and red blood cells. Copper phthalocyanin method. × 100.

FIG. 91. Human brain. Case of juvenile amaurotic idiocy. Gangliosides stored in the neurones stain brilliantly. The nucleoli are also strongly positive. Copper phthalocyanin method. × 1025.

technique preservation and staining of the smallest fat particles is excellent and it can be recommended for the purpose. Govan himself regarded his method as fitted for research rather than for routine use on account of the difficulty in preparing the solutions.

Other Solvents and Dyes

Alternative solvents have been suggested for the Sudan dyes in place of alcohol which, though they are still in the class of fat solvents, have been considered to have various advantages. Gross (1930) used Sudan IV in 50 per cent. diacetin (glycerol diacetate) and found that the smaller fat particles were well preserved, and Lillie and Ashburn (1943) used Sudan IV as a saturated solution in 99 per cent. *iso*propanol, diluting to 50 per cent. with water immediately before staining. Subsequently Lillie (1944) found Oil Red O and Oil Red 4B to be superior to the red Sudan dyes in the supersaturated *iso*propanol technique and later still (1945a and b) he recommended Oil Blue NA and Coccinel Red. These last two dyes were used from saturated solutions in 60 per cent. *iso*propanol diluted to 40 per cent. with water immediately before use, and Lillie suggested that these more dilute *iso*propanol solutions should cause smaller fat loss during staining. Chiffelle and Putt (1951) recommended propylene glycol or ethylene glycol as solvents for Sudan IV or Sudan black B. According to the authors both these glycols gave stable dye solutions which did not extract lipid particles.

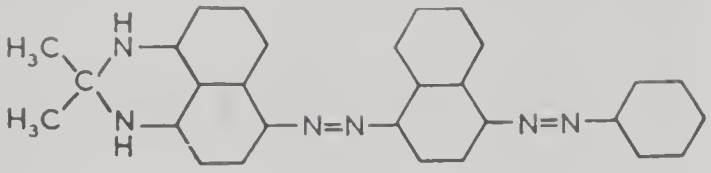
The Use of Dye Mixtures

The problem which besets the use of fat-soluble dyes for staining neutral fats remains a two-fold one. First, despite Jackson's views on the greater importance of the colloidal state of the dyes, the solvent in which they are dissolved must be used in such strength, and for so short a period of time, that removal of small fat particles is reduced to a minimum. Secondly, the solubility of the dyes in fat must be high and the colour developed must be similarly high. Kay and Whitehead (1941) tested a large number of Sudan dyes and concluded that impurities played a part in strengthening the colour imparted to fat in tissue sections. They showed that in mixtures of the Sudan dyes in alcohol each dissolves independently of the other, and the total amount of dye available for staining depends on the number of Sudans present. It is therefore good practice, in a routine fat stain, to use a solution saturated with both Sudan III and Sudan IV (commercial batches of which are themselves mixtures of a number of dyes), in 70 per cent. alcohol or in Herxheimer's mixture, in place of Sudan IV alone. This method, which was used for many years as a routine procedure in the Department of Pathology of the Postgraduate Medical

School of London, gave a satisfactory colour with neutral fats when used for so short a period as 30–60 seconds, and solution of smaller fat particles was avoided by the short staining time. Methods of this type should be replaced by the Oil Red *iso*propanol technique of Lillie for the demonstration of neutral fats in routine practice. Where the dye Fettrot is available it will be found superior to either of the red Sudans but not to the Oil Red, Oil Blue series of dyes (see Appendix 11). For research problems such techniques may be supplemented by the phosphine method of Popper and by the colloidal suspension method of Govan.

Sudan Black B

Particularly in the case of the staining of phospholipids by fat-soluble dye techniques the greatest advances have come from the use of the disazo dye Sudan black B whose formula is given below. This



dye was first introduced into histochemistry by Lison and Dagnelie (1935) and employed among other things, for staining the granules in leucocytes by Sheehan (1939), McManus (1945), Wislocki and Dempsey (1946) and Eränkö (1950). Sudan black B was fractionated by Bermes and McDonald (1956) using a 40 cm. celite-silicic acid column. They found no less than 10 fractions, respectively yellow, red, orange, green, blue, green-blue, blue, blue, blue, and black. All of these fractions stained the tissues and fraction 3 was fluorescent in ultraviolet light. It has been reported by Fredricsson *et al.* (1958) that SBB decomposes in acid solutions, and especially rapidly below pH 4. The product, which has a pK in the region of 4, is brown above this level and red below it. It can stain nucleoproteins and mucopolysaccharides and the authors consider that brown staining with Sudan black B is not to be regarded as due to lipid. The point might be tested by examination of sections in citrate buffer at pH 2·0.

This dye is unnecessary for the demonstration of neutral fats, for which the Fettrot or Oil Red or Blue dyes are more suitable, but it possesses the advantage of being far more soluble in phospholipids and to a lesser extent in cerebrosides than the above-mentioned dyes, and it will stain them in tissue sections provided they are preserved by suitable fixation. It was therefore used in a Ciaccio type of procedure (on frozen sections) by Baker (1944) and by McManus (1946). The latter employed fixation in a formalin-calcium-cobalt solution, substituting

cobalt for the cadmium of Baker's fixative for phospholipids. McManus postchromed his material where necessary and, although he used frozen sections as a routine, he pointed out that Sudan black could readily be used on paraffin sections if suitable fixatives were employed. Since formalin fixation alone preserves lipids to some extent it is always worth applying the Sudan black technique to formol-fixed paraffin sections when nothing else is available ; although a negative result is of no importance, positive results signify " formol-fixed " lipoprotein whose lipid component is usually phosphatide or cerebroside.

Baker, and also McManus, used Sudan black in 70 per cent. alcohol, and this solvent is most commonly employed despite the objections of Leach (1938), who recommended 50 per cent. diacetin. The Sudan black technique is not, of course, specific for phosphatides, but phosphatides are the most common lipids which it demonstrates if neutral fats are excluded. The result obtainable in frozen sections is illustrated in Plate IIIB, p. 289, where the phospholipids present in the cells of a so-called granular myoblastoma are seen to have great affinity for the dye. The Sudan dyes gave only faint pink staining in this case. The Sudan black technique is also useful in conjunction with the various extraction methods (outlined above) and, in conjunction with acid hæmatein, in Elftman's (1954) controlled chromation technique (see p. 313).

Metachromasia and Sudan Black B

Following the observation by Lennert and Weitzel (1952) that Sudan black B appeared reddish-violet when dissolved in octane, nonane and decane Diezel and Neimanis (1957a and b) tested 65 different organic compounds with the dye. They observed that, in addition to the aliphatic hydrocarbon series, aromatic hydrocarbons and many other substances produced a change of colour from black to red which was described as metachromasia. When viewed by polarized light lecithin, sphingomyelin and ganglioside (in alcoholic solution) produced the metachromasia, while cerebroside and cholesterol did not. In the case of lecithin the metachromasia was so strong that it was visible by ordinary light. The authors explained the metachromasia of the dye as being due to mesomerism and claimed that lipids capable of eliciting metachromasia are those which are dipoles (zwitterions). The metachromasia was not due to the production of anomalous colours since it was not abolished by rotating the preparations.

Diezel and Neimanis tested the dye on paper chromatograms with various solvents and noted changes in the colour of the front. They did not suggest that these might be due to different fractions (cf. Bermes and McDonald). Although Sudan black B metachromasia was considered to be a useful test for the zwitterion lipids I believe that

further examination of the mechanism is necessary before such an interpretation of the effect observed in tissue sections can be fully accepted.

Acetylated Sudan Black B

Observing that ethylation of fat solvent dyes could prevent or diminish staining of the leucocyte granules (stable sudanophilia) Lillie and Burtner (1953) acetylated a number of disazo and other dyes including Sudan black B. They found that the new dye would no longer produce stable sudanophilia but that the staining of fats was unimpaired. Lillie (1954) noted that the acetylated dye gave less background staining and other workers (Casselman, 1954a) have made similar observations. This author (1954b) gave details of an improved method of acetylation using an equivalent of acetic anhydride in diethyl ether (Kaufmann, 1909) in place of Lillie's excess acetic anhydride in pyridine.

Most workers will probably feel that under the staining conditions usually employed the results with unacetylated Sudan black are sufficiently good for most purposes. Moreover, in view of the fractionation tests mentioned above, one wonders how many of the 10 fractions of the dye are successfully acetylated.

The Propylene Glycol Method

The Sudans and other lipid-soluble dyes are far more soluble in propylene glycol than in alcohol or acetone, and staining in 0·5 per cent. solutions of dye becomes perfectly feasible (Chiffelle and Putt, 1951). Using Sudan IV, Fettrot (Ciba), or Sudan black B, as saturated solutions in propylene glycol, I was able to confirm the original authors' findings. No tendency to the extraction of finer lipid particles by the solvent is observed and the intensity of staining of neutral fat particles is far greater than with the usual alcoholic solvents. In addition, a large number of phosphatide-containing structures are often intensely stained, including the mitochondria and even small particles which are perhaps microsomes. Structures in which lipids cannot usually be demonstrated, such as the prickles of the prickle-cell layer of the epidermis, are also stained by this method.

A modified propylene glycol method is given in Appendix 11. It is recommended for use where demonstration of the total lipid content of cells is required, and as a routine fat stain. If it is used for the latter purpose some acclimatization is necessary since so much more lipid is shown up than by the usual techniques using acetone, alcohol or *iso*propanol. Sudan black B in propylene glycol is particularly good for staining chromated lipids produced by Elftman's method. Ethylene glycol may be used as an alternative solvent.

Marchi Methods for Degenerating Myelin

The original method of Marchi (1892) involved fixation of blocks of tissue for 3–4 weeks in dichromate before impregnation with his OsO_4/CrO_7 mixture. Modern refinements of the technique (Swank and Davenport, 1934 ; Glees, 1943) have involved abandonment of the original fixation in dichromate and the substitution of other oxidizing agents ($NaIO_3$, $KClO_3$) in the impregnating mixture. It is usually considered (Weisschedel and Jung, 1939) that treatment with oxidizing agents prevents the unsaturated fatty acids of normal myelin from reducing OsO_4. Since degenerating myelin can still reduce OsO_4 after oxidation it must either contain more unsaturated groups than can be inactivated by the oxidant or reducing groups of a type not susceptible to oxidation. Johnson *et al.* (1950), Brante (1949), Burt *et al.* (1950) and Mannell (1952) have made extensive studies of the chemical changes in degenerating myelin. It is agreed that the myelin lipids (cerebroside, cholesterol and sphingomyelin) change very little during the first 8 days after section of a nerve and that they then decrease rapidly and coequally between the 8th and the 32nd day. There is thus, as pointed out by Wolman (1956a and b) no chemical explanation for the histochemical findings in degenerating myelin. Wolman therefore tested a number of pure substances with Marchi-type procedures in order to determine, if possible, the type or types of material which might be responsible for the reaction. He found reduction only with unsaturated fatty acids and with pyrogallol, cysteine, glutathione and heparin. Pretreatment with dichromate slowed the reaction with the first of these but increased the reaction with heparin. Wolman suggested, on the basis of these studies, that the normal myelin sheath contains acidic mucopoly saccharides and that these are liberated during demyelinization. Using his Bi-Col method (Chapter IX, p. 258, and Appendix 9, p. 837) he observed a marked increase in red (gold) staining which was maximal 2–4 days after transection of the nerve. This was interpreted as being due to the presence of a polyuronic polysaccharide which was weakly Marchi-positive. A strongly acidic polysaccharide such as heparin would have taken the blue (iron) stain of the Bi-Col mixture. From the 6th–8th day onwards, rising to a maximum on the 12th day, a non-acidic polysaccharide was present which Wolman considered as a derivative of the polyuronide. This was strongly Marchi-positive.

Opposition to Wolman's view has been expressed by Adams (1958) who points out that the Marchi-positive component of degenerating myelin is soluble in acetone and behaves more like a lipid than a carbohydrate. He has offered an alternative explanation for the Marchi-negative quality of normal myelin.

According to Adams normal myelin has an affinity for water, due

to the presence of phospholipids, and it is therefore permeable to water-soluble oxidizing agents such as chlorate. Degenerating myelin has no such affinity. Furthermore Adams quotes the work of Zelikoff and Taylor (1950) who showed that OsO_4 in an OsO_4/chlorate mixture acts as an oxidation catalyst rather than as an oxidizer. According to this theory it can accelerate the oxidation of various compounds without reduction to OsO_2.

Tests carried out by Adams on myelin lipids *in vitro*, and on oleic acid and cholesterol oleate, apparently confirm these views and suggest that the hydrophobic properties of degenerating myelin may be related to the formation of cholesterol esters during the chemical degradation of myelin.

Contrary to established opinion Smith (1951, 1956), and her colleagues (Smith *et al.*, 1956), maintain that material long stored in formalin can be used for Marchi preparations, particularly if the stage of clearing is avoided. The more usual view (Swank and Davenport, 1934 ; Mettler and Harada, 1942) is that prolonged formalin fixation causes the normal myelin sheaths to stain positively and that fixation in formalin alone should not be continued for over 24 hours. Smith and her colleagues found that Marchi-positive material was at first extracellular (up to 10 weeks after injury) and then progressively intracellular. The extracellular material could be stained for about 2 years but after this most of it became negative. The intracellular material stained strongly after very long periods of storage in formalin.

Marchi methods must be performed on blocks of tissues if aqueous mixtures of the Swank-Davenport type are used. Wolman (1956b) has shown, however, that if staining is carried out in non-aqueous media such as CCl_4 the method can be applied to paraffin sections mounted on slides. In Appendix 11, p. 853, details of the Swank-Davenport procedure, and also of Wolman's procedure for paraffin sections, are given.

Chemical and Other Methods for Lipids

Osmic Acid (Osmium Tetroxide) Methods

The reduction of osmium tetroxide, in weak aqueous solutions, has long been used as the basis of methods for demonstration of lipids. Lison (1936) made it quite clear that the conditions under which osmium tetroxide is allowed to react have a great influence on the final result. Pure OsO_4 is blackened by all tissue elements but this blackening does not occur if an oxidizing agent is used simultaneously. Primary blackening with OsO_4 is considered to be due to the presence of unsaturated fatty acid (oleic acid) but the phenomenon of secondary blackening, which takes place when fats giving a negative primary

reaction are subsequently treated with alcohol, remains inadequately explained. Lison (1936) considered that osmium tetroxide should not be used in the histochemical study of lipids, since interpretation of the results was subject to very great error and he still considers (1953) that the reaction is unsatisfactory from the histochemical point of view. Cain (1950) did not agree with this exclusive point of view, but supported a limited use of osmium tetroxide in histochemistry. He maintained that if it was allowed to act on frozen sections, fixed in formol-calcium for 6 hours, it could be regarded as specific for reducing lipids, provided that only structures known to contain lipid were considered.

Metallic Mordant Methods

Ciaccio Method I. Ciaccio (1909) developed a method depending on the simultaneous treatment of fresh or formol fixed tissue with formalin and dichromate, followed by prolonged postchroming and by paraffin embedding. This treatment rendered certain lipid substances resistant to extraction by the usual processes of embedding and they could be stained, in paraffin sections, with saturated 70 per cent. alcoholic solutions of Sudan III or Sudan IV. In the original technique myelin stained red and "Ciaccio lipids" orange. In his original description Ciaccio included among the positive-staining lipids both saturated and unsaturated phospholipids (lecithin, kephalin and myelin) and mixtures of cholesterol and cholesterol esters with oleic acid. Kaufmann and Lehmann (1926), however, concluded that all Ciaccio-positives were unsaturated fats and that the reaction was specific for these.

Cain (1947a and b) considered that paraffin embedding could not be regarded as an efficient procedure for the removal of non-chromated lipids and Lison (1953) could place no confidence in the reaction although he observed that it was better when carried out with Sudan black B in place of Sudan III or IV. Although the original Ciaccio method is not now used in histochemistry the most important developments are derived directly from it. I refer to procedures using direct chromation (without previous or concomitant fixation with formalin) followed by staining with Sudan black B or hæmatoxylin mixtures.

The original Ciaccio procedure had two major disadvantages ; first, the necessity for prolonged treatment with dichromate and second, the poor final colour developed. Lillie and Laskey (1951), recognizing these features, developed a modified Ciaccio method using formol-calcium fixation followed by Sudan black B or Spirit blue. This method should not be called a Ciaccio procedure, but it is a useful variant of McManus' (1946) Sudan black method and it is interesting for its use of the Feulgen reaction as a nuclear counterstain.

Smith-Dietrich Type Methods. The original method of Dietrich

(1910) for lipins depended on the treatment of blocks of tissue with formaldehyde-dichromate mixtures by which the lipins were oxidized and combined with chromium. Frozen sections were cut and the presence of combined chromium was subsequently shown by the black colour produced by combination with acid hæmatoxylin. The class of lipins included the phospholipins, or phosphatides, with which we are concerned in this section, and the galacto- and glycolipins, or cerebrosides, considered in the section below. Chromation was originally carried out at 37°, but Kaufmann and Lehmann (1926) considered a temperature of 60° necessary and subsequent workers have followed this precept. Lison (1936) believed that a black colour with the Smith-Dietrich test indicated the presence of lipins, but that a grey tint was given by many fats and could not be regarded as of diagnostic value. A negative test, moreover, was not considered to exclude lipins. Baker (1944), by means of *in vitro* tests, showed that kephalin and sphingomyelin both stained black by the Smith-Dietrich procedure, as did lecithin when combined with other (Smith-Dietrich-negative) lipids. Lecithin alone did not react.

The Acid Hæmatein Method. The principle of the Smith-Dietrich method was used by Baker (1946b) as the basis of his acid hæmatein test for phospholipins. After fixation of tissue in formol-calcium, prolonged chromation is carried out (at 22° and 60°) in a dichromate-calcium mixture. Frozen sections (gelatin-embedded) are subjected to further brief chromation before staining in a freshly prepared and oxidized acid hæmatein solution. Differentiation is carried out with a borax-ferricyanide mixture. Baker gave a list of substances and their reactions by his method, of which the following is an incomplete summary :

Fibrinogen	pale dirty blue.
Collagen	negative.
Mucin	dark blue.
Hæmoglobin	grey.
Egg lecithin	dark blue or blue-black.
Brain lecithin	
Kephalin	
Sphingomyelin	
Nucleoprotein	dark blue.

Baker (1947) tested the reaction of purified galactolipin and obtained a negative result. He therefore concluded that, among lipid substances, the reaction was specific for phospholipids. In order to distinguish between these and other non-lipid substances giving a positive reaction, he introduced his pyridine extraction method which has already been considered in the section on extractive techniques.

Cain (1947a) conducted an examination of Baker's acid hæmatein method and concluded that the test was specific for phospholipins provided that only a definitely positive result was considered. Very pale blue and greys might be caused by other lipids which, if present in very large masses, might even show as medium to dark blue granules. Cain explained the mechanism of the test as follows:

(1) Phospholipids are not fixed by formol-calcium but are restrained from passing into solution by the calcium, which plays no other part.

(2) Phospholipids combine readily with chromium salts and are thereby rendered insoluble and mordanted as well.

(3) On staining, blue and brown colours are formed when the dye attaches itself to chromium in the various tissue components.

(4) On differentiation, some brown and most blues, particularly those in substances which contain phosphoric acid, remain nearly fast. Most browns and the weak blues are reduced or removed entirely, and for this reason **the period of differentiation must not be shortened.**

Since the specificity of the test depends on the relatively greater affinity of phospholipins for the mordant, by comparison with other lipids, the **period of chromation must not be lengthened.** It followed from Cain's views, and from other considerations mentioned above, that Baker's method was a relatively highly specific one for the histochemical demonstration of phospholipids. As such, it supplanted the older Smith-Dietrich method from which it was derived. Lison (1953) considered that substances giving a positive reaction before and a negative reaction after pyridine extraction could be regarded as lipines but commented that insolubility in pyridine did not mean non-lipid since protein-bound lipids might be insoluble. This is certainly true as noted by Almeida and Pearse (1958). Moreover I cannot feel certain that cerebrosides are not capable of giving a positive reaction since they are strongly Weigert-positive *in vitro* (Edgar, 1956) and some samples give a positive Baker's test, reversible by pyridine. A further qualification in the interpretation of the acid hæmatein reaction must be introduced. There is evidence that preservation of lipids by formol-calcium is inadequate and Baker's method will therefore demonstrate only relatively insoluble phospholipids and, perhaps, those which are bound to protein. In his original papers Baker noted some tissue components which stained positively only *after* pyridine extraction, among them chromatin and nucleoli. Staining of the latter was attributed to phospholipid by Baker but since it is reversible by means of ribonuclease it may possibly be of non-lipid origin.

Details of the method are given in Appendix 11, p. 848 and the results are illustrated in Fig. 88; red cell envelopes and myelin sheaths are strongly stained.

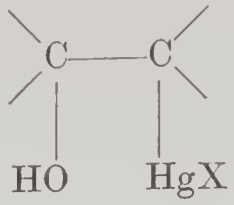
Controlled Chromation. Simultaneous oxidation, chromation, and

fixation of lipids by means of dichromate or chromic acid has been in constant use for about 100 years since Müller (1859) introduced the principle into histology. It is an essential part of all the so-called Smith-Dietrich procedures described above. Recently Elftman (1954) has drawn attention to the necessity for control of all the variable factors, time and temperature of incubation and concentration and *p*H of the reagent. He regards the last as the most important. After experimenting with these variables Elftman selected 2·5 per cent. dichromate at *p*H 3·5 and 56° for 18 hours as his standard procedure for blocks of tissue, followed by paraffin embedding and staining of sections with Sudan black B in ethylene glycol and with hæmatoxylin. Structures stained by both methods are considered to be phospholipids. Non-chromated lipids will be stained by Sudan black B only and non-lipid materials by hæmatoxylin only. When using the latter Elftman avoids the classical stage of differentiation in borax-ferricyanide by adding low concentrations of ferricyanide to the staining solution.

I believe that the principle of controlled chromation represents a real advance in lipid histochemistry and that, coupled with Sudan black B in ethylene or propylene glycol it should be used as a routine method for histochemical studies. If paraffin sections are employed it may not be necessary to use hæmatoxylin control since non-chromated lipids will probably be mainly absent. If fresh frozen cold microtome sections have been used, however, this type of control is essential unless it is replaced by one of the extractive methods. Details of Elftmann's method are given in Appendix 11, p. 849. Obviously a good deal of work remains to be done before the controlled chromation method becomes a precise tool for the differentiation of various classes of lipid.

Okamoto's Mercuric Nitrate Method for Phospholipids. The original account of this method was published by Okamoto *et al.* (1947) in the third of a series of papers in the Japanese language dealing with lipid techniques. The first description in English was given by one of the original co-authors (Ueda, 1952) in a paper describing the application of the method to 6 cases of Gaucher's disease.

The rationale of the method is not given in Ueda's paper nor is it discussed by Diezel (1954, 1957) who used the method extensively in his studies of the lipid storage diseases. As in the case of chromium salts, the effect of mercuric mercury cannot be demonstrated with reagents which fail to demonstrate the mercury-lipid complex. Among these is Naphthochrome green B (see Chapter XXIV, p. 689). Diphenylcarbazone is in fact the only reagent with which I have succeeded in making the lipid complex visible. It is possible that the reaction is based on combination of divalent mercury with unsaturated C-C bonds of the type:



These are described by Douglas (1956) who gives several references to the process. Alternatively the metal may combine with the oxidation products of olefine groups.

From the practical point of view the method depends on the treatment of thin formalin-fixed sections, after prior extraction with acetone, with alcoholic mercuric nitrate at 0° to 4°. After washing, unbound mercury is removed with potassium iodide and the lipid-bound mercury is then demonstrated by means of diphenylcarbazone. A violet colour is given by phospholipids and cerebrosides and in order to separate these two types of material the authors proposed their cold pyridine extraction, performed after staining. Their claim that this removed only cerebrosides has not been sustained by the investigations of Edgar and Donker (1957). Further claims that subsequent ether extraction would leave only sphingomyelin are also unsubstantiated. The results of the method are illustrated in Fig. 89, p. 305. I consider that the principle involved in this method may be an important one and that efforts should be made to elucidate the mechanism. If this can be done successfully a useful technique will be added to the rather poor selection at present available for demonstrating phospholipids.

The Phosphomolybdic Acid Method. Choline-containing lipids can be demonstrated on paper chromatograms by forming insoluble choline-phosphomolybdic acid complexes which are subsequently rendered visible by conversion to molybdenum blue. This mechanism is to be distinguished from that of the method for inorganic phosphates (Chapter XXIV, p. 710) in which water soluble phosphomolybdates are formed by treatment with ammonium molybdate.

Landing *et al.* (1952) successfully demonstrated choline-containing lipids in tissue sections in this way. They were able to stain sphingomyelin deposits in Niemann-Pick's disease and the cerebrosides of Gaucher's disease (in spite of their presumed lack of choline) and I have used their method to show the gangliosides in various cases of amaurotic idiocy. Landing and his co-authors could prevent the staining of sphingomyelin and cerebroside by brief treatment of sections with a number of organic solvents, especially ethanol, pyridine and acetone-ether mixtures. Chloroform, xylene and acetone were ineffective. They considered that the results of these extractions indicated that the lipids concerned were present as lipoprotein complexes and emphasized the inapplicability in histochemistry of any differential extraction scheme based on the known solubility characteristics of

purified lipids. When the phosphomolybdic acid method is applied to paraffin sections much more intense staining of various interstitial tissues is noted and a number of procedures, such as treatment with dichromate or 70 per cent. ethanol, or with acetone for long periods, will enhance this staining. Landing *et al.* believed these effects to be due to the release of potentially active compounds normally present in bound form. The phosphomolybdic acid method is particularly applicable to fresh cold microtome sections, free-floating or mounted on slides, but even with these the results may be difficult to interpret. Details of the method are given in Appendix 11, p. 851.

Bound Lipids. Although these are not to be classified as choline-containing brief mention of some techniques used for their demonstration may be appropriate at this point.

A modified Sudan black B technique for masked or bound lipids in blood films was described by Ackerman (1952). This involved pretreatment of the films with various organic acids (acetic, citric, oxalic and formic) and subsequent staining with a matured 70 per cent. alcoholic solution of Sudan black B. The reaction of lymphocyte mitochondria was considerably enhanced and the platelets stained positively after acid treatment. The staining of masked lipids has also been studied by Brolin (1952). A further application of Sudan black B to the staining of bound lipids in tissue sections was described by Berenbaum (1954) who found that after long washing or exposure to dry heat, *inter alia*, many presumably lipid-containing structures could be stained with Sudan black in acetone or with hot (burning) ethanolic Sudan black. The staining of lipoproteins by methods such as these has now been more fully considered by Berenbaum (1958). Details of these techniques are given in the Appendix to this chapter.

The Copper Phthalocyanin Methods. Copper Phthalocyanin (CuPC) dyes were first used in lipid histochemistry by Klüver and Barrera (1953, 1954) who employed Luxol Fast blue B (Dupont) for staining myelin sheaths in paraffin sections. This dye is the amine salt of a sulphonated CuPC which has the same basic structure as Alcian blue (see p. 259). Dyes of this type, which can be represented by the symbols (CuPC) SO_3H.Base, may have from 1 to 4 sulphonyl groups per molecule and one of a number of different bases. Their staining characteristics with regard to lipids depend not so much on the type of base employed as on their solubility in organic solvents. The nearest British equivalent to Luxol Fast blue is Methanol Fast blue 2G (I.C.I. Ltd.) and this performs in an essentially similar manner.

Klüver and Barrera (1954) carried out a number of experimental studies on formalin-fixed sections, using Luxol Fast blue in combination with various counter-stains. They found that pretreatment of fixed sections with organic solvents never abolished staining. If these

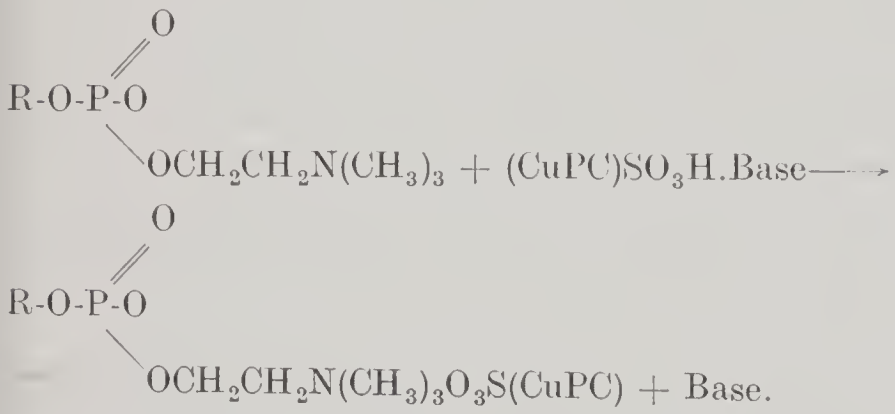
solvents were applied to unfixed blocks of brain tissue, however, myelin was effectively prevented from staining. No attempt was made by Klüver and Barrera to explain the staining of myelin in chemical terms. I therefore tested a number of lipid materials *in vitro* with Luxol and Methanol, with the results given in Table 21, below.

TABLE 21

In vitro Tests with Copper Phthalocyanins

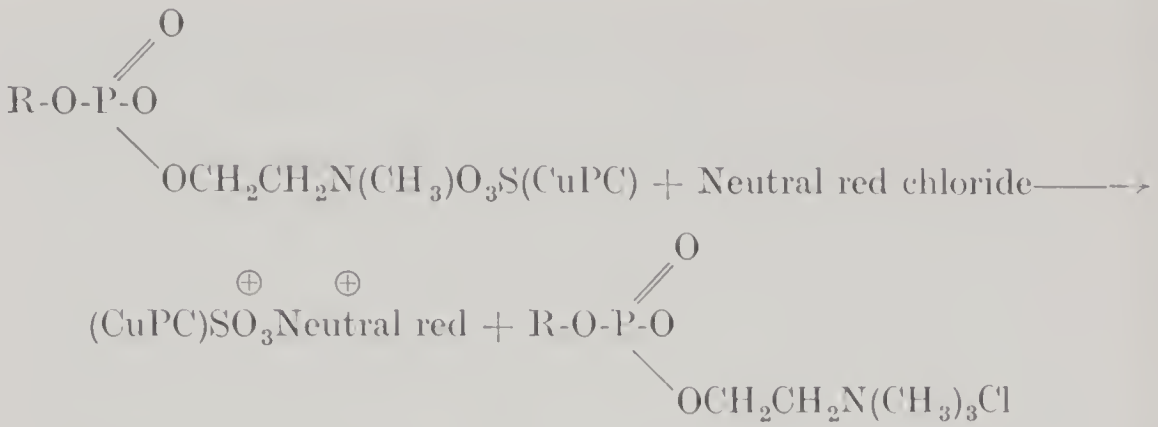
Substance	Solvent	Result	Type of Ppt.	Time
Lecithin .	Warm EtOH	ppt.	Dark Blue	Rapid
Kephalin .	Warm $CHCl_3$	nil	——	——
Sphingo-myelin .	Warm $CHCl_3$/MeOH	ppt.	Dark Blue	Rapid
Cerebroside .	Cold pyridine	ppt.	Paler Blue	Slow
Phosphoryl choline .	30% EtOH	ppt.	Dark Blue	Slow

As a result of these findings I suggested (1955) that since formation of the characteristic dark blue precipitates was confined to choline-containing compounds the following mechanism of staining (in alcoholic solutions) might apply.



Effect of Neutral Red. Although Klüver and Barrera used neutral red as one of their counterstains they did not record any unusual effects. I found, however, that the dark blue CuPC-lipid complex in the tissues was converted by aqueous neutral red into a black or blackish-purple one. This effect could be produced *in vitro* but only in aqueous media in which the insoluble CuPC-lipid complexes had to be suspended. With alcoholic solutions of the dyes neutral red formed a black complex (or salt) of similar colour to the one mentioned above. These mechanisms were explained as follows : (I) in alcohol,

$(CuPC)SO_3H.Base + Neutral\ red^{\oplus}\ Chloride^{\ominus} \longrightarrow (CuPC)SO_3^{\ominus}Neutral\ red^{\oplus} + Base\ HCl$ and 2), in water,



While the first of these two equations is probably true, if the second were also true it should be possible to recombine the liberated phospholipid with a further phthalocyanin molecule. No such effect could be produced and I believe, therefore, that the reaction of Neutral red is with SO_3H.Base groups left uncombined after formation of the CuPC-lipid complex.

It was always evident that CuPC would stain certain non-lipid tissue components such as nuclei, nucleoli, and calcium deposits though the latter, of course, provide no problems in distinction. For this reason further studies were carried out (Pearse and Almeida, 1958) with lipid and non-lipid materials either on filter paper or on Coujard-type slides. Alcoholic solutions of Luxol Fast blue or Methanol Fast blue stained all the lipids tested, including ganglioside, with the single exception of sphingomyelin. Of the non-lipid substances only protamine base and protamine sulphate were stained. Almost certainly the reaction of these last two substances was due to their arginine content. This amino-acid gives a dark blue precipitate with (CuPC)SO_3H.Bases, and with various sulphonated CuPC's in solution, but it is removed by processing in the paper and Coujard tests.

My present view of the mechanism of CuPC staining of lipids and lipoproteins is as follows : (1) In fixed tissues (especially paraffin sections) lipo-proteins rather than lipids are responsible for staining. (2) The mechanism is that of an acid-base reaction with salt formation where the base of the lipoprotein replaces the base of the phthalocyanin. (3) With the majority of lipoproteins the reaction does not take place unless the dye is dissolved in a lipid solvent. (4) Sphingomyelin stains only when it is insoluble in the dye bath, as when chloroform is used for this. (5) The chemical basis of ganglioside staining remains an enigma. The constantly observed staining of the nucleoplasm of a small percentage of nuclei in most tissues, and of the nucleoli in others, may not be due to phospholipid. However, it is worth noting that Chayen and Gahan (1958) have found in calf thymus nucleohistone the equivalent of 6 per cent. sphingomyelin (as $CHCl_3$-soluble nitrogen).

Since the staining of elastic tissue with CuPC (Chapter VII, p. 168) has also been ascribed to the presence of sphingomyelin there may be some link between the two.

From the practical point of view the CuPC method provides an excellent stain for myelin in formalin-fixed frozen sections and in paraffin sections after a variety of fixatives. As shown by Almeida and Pearse (1958), the sensitivity of the method for myelin lipids is high, being equal to that of the acid hæmatein method except, in developing rabbit brain, at the stage where sphingomyelin alone is present in the white matter. Developing myelin sheaths are shown in Figs. 90 and 91, p. 305, where the abnormal type of ganglioside stored in the neurones in cases of juvenile amaurotic idiocy can be seen to stain very strongly. Plate IIIc, p. 289, shows the application of the method to the demonstration of phospholipid stores in the egg of a marine gastropod *Buccinum undatum*. Practical details of the method are given in Appendix 11, p. 857.

The Copper Method for Fatty Acids (Fischler). Benda (1900) first demonstrated the affinity of fatty acids for heavy metals by forming green copper salts with cupric acetate, and Fischler's (1904) technique, based on this principle, depended first on the formation of calcium soaps from fatty acids. Tissues were fixed in formalin containing calcium salts, frozen sections were cut and then mordanted in a saturated copper acetate solution. They were stained with Weigert's lithium hæmatoxylin and differentiated in a dilute solution of Weigert's borax ferricyanide mixture. According to Lison (1936), iron and calcium salts and "certain non-fatty tissue elements" were also coloured grey or black. Mallory (1938) pointed out that hæmoglobin was strongly stained but that this objection could be overcome by differentiating in borax-ferricyanide until the red cells were colourless. He apparently overlooked the fact that red cells are stained because of their lipoprotein envelopes. Mallory also observed that errors due to calcium might be avoided by pretreatment with dilute HCl and those due to iron by pretreatment with 5 per cent. oxalic acid. Lison, however, maintained that the reaction was not specific for fatty acids and that Fischler's method should not be used in histochemistry for the demonstration of these substances. In spite of these views, however, details of the technique are given in Appendix 11, p. 858. In tissues where fatty acids are known to be present, as when they have been injected for instance, Fischler's method gives reliable results. Where the demonstration of fatty acids in small quantities is required the result must be interpreted with extreme caution.

An alternative method for fatty acids, making use of the same principle, was developed by Okamoto, Ueda and Kato (1944). In this variant of Fischler's technique the copper soaps resulting from the

first stage of the reaction are made visible by means of *p*-dimethylaminobenzylidene rhodanine. Details of this method are also given in the appendix though I am doubtful of its efficacy. In several tests carried out on normal tissues I was unable to obtain a positive reaction.

Methods for Cholesterol and its Esters

The Liebermann-Burchardt Reaction. This reaction, considered at one time to be specific for cholesterol and cholesterol esters, has been considered by Bierry and Gouzon (1936) to be a general reaction for unsaturated steroids and by Everett (1947) to indicate the presence of diols formed by mild oxidative procedures. Boscott and Mandl (1949), however, applied the reaction to pure samples of dehydro-*iso*-androsterone, progesterone and deoxycorticosterone acetate without obtaining the characteristic blue-green colour. They obtained instead colours varying from yellow to orange.

Kent (1952) tested 24 compounds, including cholesterol and cholesterol esters, by the Liebermann-Burchardt test and by the Schultz histochemical variant. All the compounds tested produced a reaction with the former but only in the case of linoleic acid, carotene, vitamin A and ergosterol could the blue-green colour be confused with that given by cholesterol and its esters. With the Schultz test only carotene produced the same blue colour as cholesterol and its esters. In practice, therefore, the Schultz test can be regarded as highly specific for cholesterol and cholesterides although its specificity is not absolute.

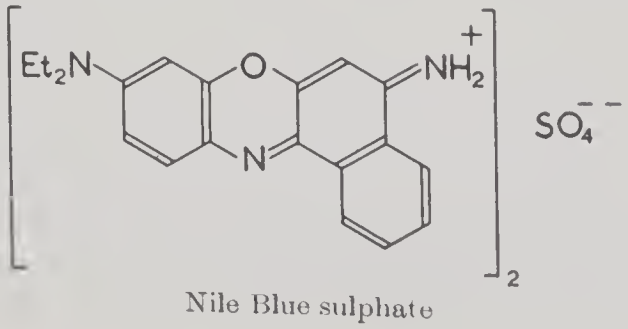
The original histochemical modification, which was described by Schultz (1924, 1925) and by Schultz and Löhr (1925), necessitated previous oxidation of the cholesterol either by exposure to light for at least four days or by treatment with 2·5 per cent. iron alum at 37° for 2 days. Sections were then mounted on slides and treated with a mixture of equal parts of glacial acetic acid and concentrated sulphuric acid. They were examined in this medium, under a coverslip. Previous oxidation was omitted in the modification introduced by Romieu (1927). In this technique, the action of a few drops of concentrated sulphuric acid was arrested by the addition of a similar amount of acetic anhydride. After washing with the latter reagent, the preparation was examined under a coverslip. The initial blue, violet or red colour resulting from either of these modifications of the Liebermann-Burchardt reaction is not diagnostic. If cholesterol or cholesterol esters are present the colour changes after a few seconds to bluish-green, and this colour alone is to be regarded as specific. After a longer period the whole section becomes brown. It is essential that the reagents used for this test should be as pure as possible. Most authors now regard the stage of oxidation and mordanting with iron

alum as essential. Baker and Selikoff (1952) carried out this stage for 3 days before proceeding to the sulphuric acid—acetic anhydride stage and Weber *et al.* (1956) mordanted their sections for 7 days, in buffered ferric ammonium sulphate. They found that a final pH of 2 gave the best results, and their modification appears in Appendix 11, p. 860. A slightly different type of reaction for cholesterol was used by Okamoto, Shimamoto and Sonoda (1944). This depended on the development of a green or bluish-green colour when formalin-fixed sections were treated with a 30 per cent. solution of sulphuric acid containing iodine and potassium iodide. Preparations made by this method are no more stable than with the various Schultz reactions but the reagent is less damaging to the sections. Its specificity has not been tested, to my knowledge.

The Bismuth Trichloride Method. A method for demonstrating cholesterol has been reported by Grundland, Bulliard and Maillet (1949) which made use of the colour developed when tissues fixed in an alcoholic digitonin solution were treated for 24 hours with bismuth trichloride in anhydrous nitrobenzene. This was adapted from the colorimetric method of Clark and Thompson (1948) for steroids. After development of the colour the tissues were cleared in a bath of glycerol monostearate, embedded in paraffin and sections were cut in the usual manner. This method seemed to possess a definite advantage over the more commonly employed Liebermann-Burchardt procedure, in that it was far less destructive to the tissues. For this reason localization would be expected to be more accurate, but the specificity of the method (given in Appendix 11) has not yet been established and my own results have always been patchy and lacking in clarity.

Methods for Acidic Lipids

Staining with Nile Blue Sulphate. According to Lorrain Smith (1908) his Nile Blue sulphate method stained neutral fats red, fatty acids dark blue and nuclei, elastic tissue and cytoplasm pale blue. The essential part of this method was the preparatory hydrolysis of the blue dye salt with dilute sulphuric acid. This process yields a small percentage of the free base (oxazine) which is red, and of the oxazone derivative (Nile Red) which has a similar colour. Hydrolysed solutions of Nile



Nile Blue sulphate

Blue, therefore, consist partly of the blue salt and partly of the red free base and the red oxazone. Lorrain Smith considered that the red base dissolved in glycerides while the blue salt dissolved in the fatty acids. Lison (1936), however, concluded that a rose colour by the Nile Blue sulphate method certainly indicated the presence of unsaturated glycerides, while a blue colour had little or no significance and did not even signify the presence of fat. The method as originally proposed was not altogether satisfactory and interpretation of the results, especially of the blue staining part, was difficult. Some authors, Knaysi (1941) for instance, demonstrated that the hydrolysis of fat by bacteria could be shown equally well by other dyes such as neutral red or methylene blue.

Mechanism of Staining. The experimental basis of the Nile Blue sulphate method has been more firmly established by the work of Cain (1947b). This author discussed the results of Kaufmann and Lehmann, who used Nile Blue sulphate on various lipid substances incorporated in elder pith, and he concluded: (1) That substances in the solid state, except "greases," do not colour with aqueous Nile Blue solutions in any way whatsoever. (2) That triglycerides, or mixtures of them, free or dissolved in hydrocarbons, are coloured red by the oxazone and much less so by the free base (oxazine). (3) That fatty acids, if liquid, colour blue with solutions of the oxazine (by forming salts) and blue with dilute Nile Blue sulphate solutions. With concentrated Nile Blue solutions, on the contrary, only a slight change towards blue is seen except with oleic acid which colours fairly strongly. (4) Lecithin (perhaps all phosphatides) stains deep blue, when solid, with the oxazine or with concentrated or dilute aqueous Nile Blue solutions. It is not affected by the oxazone. (5) Cholesterol in solution does not stain at all with Nile Blue.

Cain's Technique using Nile Blue Sulphate. Cain evolved a new technique with Nile Blue sulphate based on the following observations. Neutral lipids will dissolve out of aqueous solutions of Nile Blue only the oxazone and free base (both red in colour). Acidic lipids will dissolve the oxazone and combine with the free base to form blue lipid-soluble compounds. As Lison demonstrated, 1 per cent. or stronger Nile Blue solutions are not hydrolysed and Cain found that only lecithin and oleic acid would colour blue to any extent with 1 per cent. Nile Blue. In order to make full use of the histochemical possibilities of Lorrain Smith's method one must employ both the oxazone and the oxazine and the lipids under investigation must be liquid. The new technique employs frozen sections from material fixed in Baker's (1944) formol-calcium mixture, postchromed if necessary, one stained in 1 per cent. Nile Blue at 60° and the other in 1 per cent. Nile Blue at 60° followed by staining in 0·02 per cent. Nile Blue at the same temperature. If there

is no difference between the two sections the first is dispensed with. Identification of lipid structures is made in control sections stained with Sudan black B and, according to Cain, the new method allows distinction between neutral lipids (esters and hydrocarbons) and acidic lipids (lecithins). It cannot be used for the demonstration of fatty acids. Cain's Nile Blue method, which is a useful one under some circumstances, appears in Appendix 11, p. 856.

Menschik's Method using Nile Blue Sulphate. Menschik (1953) introduced into the technique a series of differentiations which he claimed could make the technique specific for phospholipids. The first of these, with acetone at 50° was followed by a second stage of differentiation with weak acid since the author had observed that Nile Blue-stained phospholipids were weakly acid-fast, while similarly stained proteins were not. This method, which is given in Appendix 11, p. 856, represents the best use to which Nile Blue has yet been put as far as histochemical practice is concerned. Menschik's tests for the specificity of the method included staining of the pure substances, with and without extraction with lipid solvents, and he concluded that it was as specific as the acid hæmatein method. It is certainly far easier to carry out. Whether the Nile Blue method is capable of demonstrating protein-bound phospholipid is open to doubt but the results, which are clear cut and easily obtained, justify extended use of the method.

Acid Solochrome Cyanine. This dye has been described in Chapter V, p. 82, and also in Chapter XXIV. It is an indicator dye which stains acidic proteins (nucleoproteins) blue and basic proteins red. In mammalian tissues it does not stain mucins by virtue of their acidic groups but only by virtue of the available basic groups of the protein. They therefore stain in shades of pink to red. In unfixed frozen sections, and in sections briefly fixed in ethanol or acetic-ethanol, the lipids of the myelin sheath stain dark blue in acid solutions of the dye (pH 2·1). When the sections are extracted with lipid solvents, such as light petroleum and chloroform-methanol, or if paraffin sections are stained, the myelin sheaths stain red. This they do presumably by virtue of their protein component which may or may not be neurokeratin. Fig. 92, p. 336, shows the staining of acidic lipid with acid solochrome.

Metachromatic Methods

Feyrter's Mounting-Staining Method. Feyrter (1936) evolved his tartaric acid-thionin mixture, in the first instance, for demonstrating the metachromasia of myelin sheaths in frozen sections. The method has since become widely employed on the Continent (see Pischinger, 1943 ; and Pretl, 1948), where it is apparently regarded

as specific for lipins in the sense described earlier in this chapter. The method is carried out by mounting formalin-fixed frozen sections on slides (with egg albumin and gentle heat if necessary) and covering them with Feyrter's acid-thionin mixture. A coverslip is then applied and the preparation is ringed with vaseline to prevent evaporation. After an interval of 24 hours or more, which may be shortened by the application of moderate heat (60°), lipid materials other than neutral fat are coloured deep rose pink. They are examined with the stain *in situ*, without removing the coverslip. The word "Einschluss," used in respect of Feyrter's method by Continental authors, refers to the process of covering and ringing and not, as might easily be supposed from the common translation "inclusion," to the particular bodies which are stained by the method. Even the translation "enclosure" is imperfectly descriptive and it has been suggested to me that "Mounting-Staining" might be a better and more acceptable term. I have therefore adopted it in the heading to this section. Hamperl (1950) gives some additional details of the application of the method particularly to the demonstration of lipid substances present in cells known as oncocytes, originally described by him in salivary, pituitary and thyroid glands, and in various other organs. There is no doubt that phosphatides are the principal lipid substances demonstrated by Feyrter's method and this is presumably on account of the capacity of the phosphoryl group to induce metachromasia. Distinction of Feyrter-positive lipid from non-lipid substances giving metachromasia with thionin is not usually a matter of difficulty. Interference due to the presence of small quantities of mucopolysaccharide may make it difficult to use the method for detection of small quantities of lipid combined, for instance, as lecithoproteins or other protein-phosphatide complexes.

No critical studies of the Feyrter technique appear to have been carried out in recent years although Brante (1957), while regarding the technique as "more or less specific for glycolipids or lipopolysaccharides" showed that gangliosides would produce a small γ-band in high concentrations with toluidine blue. He further showed that 0·5 per cent. ganglioside in gelatin readily produced metachromasia with Feyrter's reagent. I certainly agree that a positive Feyrter reaction can be obtained on the stored material in most of the cerebral lipidoses whatever its supposed composition and regardless of widely differing responses to other histochemical tests. It is possible that the Feyrter metachromasia of nervous tissues does not run exactly parallel to their toluidine blue metachromasia which Landsmeer (1952; Landsmeer and Giel, 1956) regards as due entirely to phosphate groups. Although the part played by sulpholipids is probably a small one it must not be overlooked entirely.

Reduction Methods

The Iodine Cyanide Method. A somewhat complex method, stated by the author to be specific for phosphatides, was described by Alsterberg (1941). This method depended on the premise that the strong oxidizing agent iodine cyanide (cyanogen iodide, ICN) gave no precipitate with silver salts unless a reducing agent was present at the same time. Lecithins and kephalins acted as reducing agents in this respect and produced a precipitate of silver iodide and silver cyanide. Other substances were not demonstrated to possess this property. In the application of the method unfixed blocks of tissue, after incubation in an iodine cyanide-silver chlorate solution for 3 days, were embedded by the combined celloidin-paraffin method and sections brought to xylene were treated with H_2S in xylene to convert the silver precipitates to brown sulphides.

Alsterberg's work was done entirely on animal brain tissues and the histological results obtained were in some cases excellent. Although I have no practical experience of the method, it seems likely, from a study of Alsterberg's experiments, that many factors influenced the deposition of silver salts which were independent of the nature of the substances present. Differences in pH between centre and periphery of the blocks, for instance, led to staining variations in the final result. It is anticipated that the difficulty of making both iodine cyanide and silver chlorate will prevent the use of the method except, perhaps, for research on the central nervous system ; it is given a place in this section solely on account of its theoretical interest, as illustrating the employment of a new principle in the histochemical demonstration of phosphatides.

Oxidation Methods

The Periodic Acid-Schiff (PAS) Reaction. The reaction of periodic acid with lipid substances has already been considered in Chapter IX and will not be further considered at this point. Due to the very wide specificity of the reaction it cannot be used primarily for the demonstration of unsaturated lipids in the phosphatide class, but it may draw attention to the presence of these when used for other purposes.

The Performic Acid-Schiff (PFAS) and Peracetic Acid-Schiff (PAAS) Reactions. The reaction of keratin, after oxidation with performic and peracetic acids, with Schiff's reagent and, *inter alia*, with methylene blue and Alcian blue, has already been described in Chapters V and VII. Here the application of the performic and peracetic acid-Schiff techniques to lipids present in the tissues is considered.

It was noted in the early stages of some work on keratin (Pearse, 1951a) that the red cell envelopes constantly gave a positive reaction

and, subsequently, a number of other structures were observed to react. Among these were myelin sheaths and certain intracellular granules contained usually, but not invariably, in macrophages. The latter were considered to belong to the lipofuscin group (see Chapter XXIV, p. 661) and the presence of lipid was the factor common to all. Materials which constantly give a positive PAS reaction, such as epithelial mucins, glycogen, cartilage, pituitary β granules and the basement membranes of the kidney, were PFAS-negative.

Since Verne (1929) had shown that mild oxidation of fatty substances containing unsaturated groups, such as lecithin for instance, would produce groups having all the reactions of aldehydes it seemed probable that a similar mechanism was responsible for the positive PFAS reaction in known lipid-containing structures. Various experiments were carried out in order to test this hypothesis, and Table 22 (Pearse, 1951b) gives the reactions obtained with red cell envelopes and myelin sheaths together with those of hair shafts (keratin) for comparison. Figs. 93 and 94 (p. 336) illustrate some of the results obtained with performic acid oxidation of the first two structures. In the first, the groups produced in red cell envelopes have been demonstrated by means of an alkaline silver solution and in the second, the groups in myelin have been shown by performance of the NAHD routine (see Appendix 12, p. 863).

TABLE 22

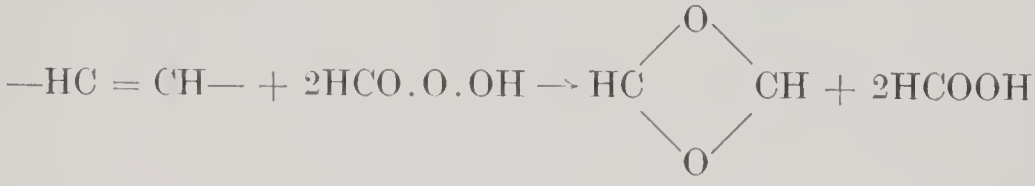
Reactions of Oxidized Lipids and Keratin

STRUCTURES	TESTS				CONTROLS	
		Performic acid			Formic acid	H_2O_2
	Schiff	NAHD	Meth. blue pH 2·6	Ammon. silver	Schiff	Schiff
Red cell envelope	+	+	−	+	+	−
Hair shaft	+	+	+	−	−	−
Myelin sheath	+	+	−	+	weak +	−

The reactions used were performic acid oxidation, followed by Schiff's reagent, by the NAHD routine (2-hydroxy-3-naphthoic acid hydrazide followed by diazotized *o*-dianisidine), by methylene blue at pH 2·6 and by an ammoniacal silver solution. The first and second reagents demonstrate aldehydes and some other groups, the third demonstrates strongly dissociating acid groups and the fourth reducing groups including aldehyde.

Since formic acid is certainly, and free H_2O_2 probably, present in

the performic acid solution in these tests, control sections were treated with the two reagents separately. With 10 per cent. H_2O_2 negative results were obtained, but with 98 per cent. formic acid a weak positive reaction occurred in myelin sheaths and a strong one in the red cell envelope. The reaction with myelin was entirely reversed by fixation in boiling chloroform-methanol for 8–16 hours, but traces of a positive reaction remained in the red cells even after this procedure, which is presumed to remove all but the most closely bound lipid from small blocks of tissue. Both red cell envelopes and myelin sheaths reduce ammoniacal silver solutions after performic acid oxidation, while keratin in tissue sections fails to do so. According to Cain (1949), aldehydes are the reducing groups most likely to be present in oxidized lipids and the reaction with ammoniacal silver was considered to have this significance. The peroxides and hydroperoxides, which Cain considered to be responsible for the plasmal reaction (Chapter XII, p. 347) of certain lipid structures, should give a positive reaction with the "Nadi" reagent (Chapter XVIII, p. 511), but this effect was not seen in performic acid-oxidized sections. Lillie (1952) working independently on the PFAS reaction in the case of ceroid pigment (Chapter XXIII, p. 666) considered that the positive result with Schiff's reagent was due to the oxidation of the ethylene groups of unsaturated lipids to aldehydes. He gave the following formula to explain the reaction :



and, by rearrangement, two aldehyde groups $(—HC = O)_2$ are produced. Lillie showed that prior acetylation, which prevents the reaction of 1 : 2-glycols with periodic acid, had no effect on the PFAS reaction of ceroid whereas bromination ($HC = CH \rightarrow BrHC.CHBr$), which has no effect on the oxidation of glycols by periodic acid, reversed the PFAS reaction completely. He concluded that ceroid contains both ethylene and 1 : 2-glycol groupings.

The PFAS reaction cannot be considered as a routine method for the demonstration of phospholipids (and other lipids in frozen sections) containing unsaturated C = C bonds. Its chief uses, at present, are as a control method for the PAS reaction and as an instrument of research into the mechanism of fat oxidation. A suggested method for carrying out the PFAS reaction on tissue lipids, together with Lillie's method for this purpose appear in Appendix 11. If a tissue component in which the PAS reaction is positive also gives a positive PFAS reaction, it is necessary to determine whether the former is due

to the 1 : 2-glycol grouping. According to Lillie, therefore, the PAS reaction is carried out after (1) acetylation and (2) bromination. A negative reaction in the first case, with a positive reaction in the second, indicate 1 : 2-glycol.

There is still no general agreement that aldehydes are produced from unsaturated lipids in tissue sections by oxidation with the two per-acids, though this view is strongly held by Lillie. Findlay (1955) carried out the PAAS reaction *in vitro* on a number of pure substances, and others, containing ethylene groups. The reaction was negative in all cases. He was also unable to extract from keratin, the most strongly PAAS-positive material with the possible exception of ceroid, any bound-lipid component. This evidence, however, does not necessarily mean that the reaction has not the significance attached to it by Lillie, when performed on tissue sections. Landing and Hall (1956) suggested alternative reagents capable of producing histochemically demonstrable products from ethylene groups of which iodoform-peracetic acid was the most promising and specific. These authors agree with Lillie's interpretation of his reaction.

The U.V.-Schiff Reaction. Belt and Hayes (1956), after carrying out a number of experiments on the Schiff reactivity of tissue lipids, evolved a method in which thin blocks are briefly fixed in formalin and then infiltrated with gum arabic. Frozen sections are subjected to long and short wave (254 mμ) U.V. irradiation for 3–4 hours and then placed in Schiff's solution. The authors claim that "there is considerable evidence that this reaction demonstrates double bonds" and I agree with this view although complete proof of specificity is difficult to obtain.

Using a 250-watt B.T.H. Mercra lamp and 2–4 hours' exposure, strong staining (with negative controls) was produced in the intestine of the larva of the waxmoth *Galleria mellonella* which has been shown by Przelecka (1956) to contain large quantities of phospholipid. Other cytochemical evidence (osmium tetroxide, bromination, PFAS) suggests that these phospholipids contain a considerable quantity of unsaturated fatty acids. Details of the method of Belt and Hayes are given in the Appendix (p. 859). It is particularly suitable for application to fresh cold microtome sections.

Reactions for Sugar Components

Modified Molisch Reaction (Diezel, 1954). In his studies on the lipid storage diseases Diezel employed a modification of the Molisch reaction for carbohydrates which gave a red colour with hexose-containing compounds (cerebrosides and gangliosides). Details of the histochemical test are given in the Appendix (p. 848). This is a heroic type of procedure, reminiscent of the older microchemical tests which

had to be interpreted in the few remaining fragments of the section. If Diezel's instructions are carried out, however, the sections remain surprisingly intact.

Modified Brückner Reaction. This reaction (Brückner, 1943), which is positive *in vitro* with pentoses and hexoses, was also modified by Diezel (1954) for use on tissue sections. A red colour is given by cerebrosides and gangliosides but mucopolysaccharides do not react. Details of the histochemical test are given on p. 848.

Modified Roe and Rice Test for Pentoses. A reaction specific for pentoses was developed by Roe and Rice (1948) in which carbohydrates of different types cause little or no interference. Applied by Diezel (1954) to tissue sections the reaction was negative with cerebrosides and gangliosides but it gave a red colour with sections of plant seeds containing pentoses. Details are given in the Appendix, p. 848.

Recommended Methods for Various Types of Lipid

A number of methods for neutral fats, fatty acids, and phospholipids have been considered in this chapter. It is concluded that no satisfactory histochemical technique for fatty acids exists, and that by staining methods alone absolute distinction between the various groups of lipids is sometimes a matter of great difficulty. Recommended methods are given in Table 23, below.

TABLE 23

Recommended Methods for Lipids and Lipoproteins

Routine (R) or Research (S)	Lipid Component	Type of Section	Fixation	Method
R	Neutral fats	Frozen	Formalin	Oil Red O in *iso*propanol (Lillie)
S	Neutral fats	Frozen	Formal-calcium Formalin	Fettrot in propylene glycol Colloidal Sudan III (Govan)
R	Phospholipids	Frozen	Formol-calcium Formalin	Phosphine 3 R (Popper) Sudan black B in alcohol
		Frozen	Formol-calcium	Nile Blue sulphate (Menschik)
		Paraffin	Formalin	Sudan black B in alcohol or in ethylene glycol
		Paraffin	Controlled chromation	Hæmatein, controlled by Sudan black B ethylene glycol
S	Phospholipids	Cold microtome	Controlled chromation	Sudan black, Nile Blue, hæmatein, copper phthalocyanin
R	Phospho- and glycolipids	Paraffin	Various	Copper phthalocyanin
R	Glycolipids	Frozen or paraffin	Various	Periodic acid-Schiff
R & S	Lipoproteins	Cold microtome	Dichromate	Copper phthalocyanin
S	Lipoproteins	Frozen	Formalin	Benzpyrene-Caffeine (Berg)
R & S	Unsaturated fatty acids (lipid-bound)	Frozen or paraffin	Formalin	Performic acid-Schiff
		Cold Microtome	Nil, or brief ethanol	U-V. Schiff
R & S	Acidic lipids	Cold microtome	Nil, or brief fixation (various)	Acid solochrome cyanine. Mounting-staining (Feyrter)

The above suggestions are given entirely without reference to the various extraction techniques, which can be used in conjunction with most of them. As stated already in this chapter, there is no reason to discontinue the use of extraction techniques. There is at present, however, a need for great caution in accepting their effects as in any way similar to those obtained *in vitro*. Until parallel chemical studies have been carried out for each histochemical extraction technique interpretations must continue to be made with reserve.

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CHAPTER XII

ALDEHYDES AND KETONES

The importance of aldehydes and ketones in histochemistry is related to two things. First, their presence in naturally-occurring substances, and second, their production in the tissues by a wide variety of chemical manœuvres commonly used as tests for one substance or another. Naturally-occurring *reactive* aldehydes do not, in fact, concern us, but *potential* aldehyde groups are widespread in two classes of material especially, namely deoxyribonucleic acids and lipids. From these they are released by very simple procedures such as mild acid hydrolysis, as in the Feulgen nucleal reaction, or by mild oxidation as in the plasmal reaction considered later in this chapter.

Stronger reactions involving oxidation commonly result in the production of reactive aldehyde groups in carbohydrates and lipids as in the Bauer reaction for glycogen, the PAS reaction for 1 : 2-glycol groups and the PFAS reaction for unsaturated HC = CH groups in lipids. In many of these cases there is room for doubt as to whether the reactive group is aldehyde (RH.C = O) or ketone $\begin{matrix} R.C=O \\ | \\ R' \end{matrix}$ since the reactions of the two are essentially very similar. The only important naturally-occurring ketones with which we are concerned are present in the ketosteroids, and many of the methods described have been applied to the histochemistry of these substances.

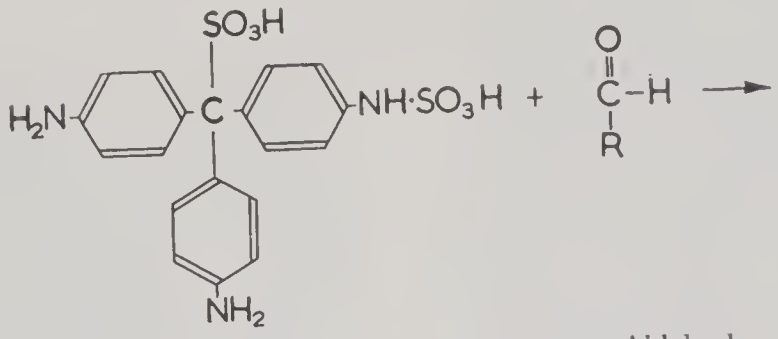
All the reactions considered in this chapter are given first under the heading of aldehyde reactions, without prejudice to their position in relation to ketones and ketosteroids. The performance of the various reactions in the latter respect will be dealt with in short separate sections. In another section full consideration is given to the plasmal reaction, not for its intrinsic importance in histochemistry but for the very great bearing which it has on the whole question of tissue aldehydes.

Positive Methods for Aldehydes

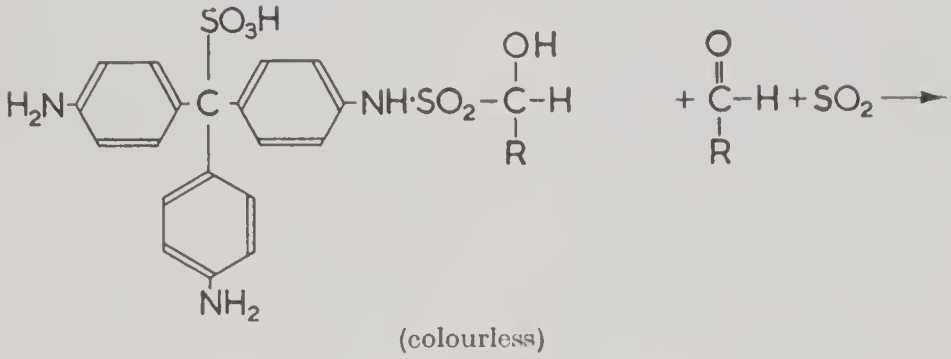
The Schiff Reaction

Enough has already been said about this reaction, in Chapters VIII and IX, to establish its primary position for the demonstration of reactive aldehyde groups. Although the mechanism of the reaction

is not known with certainty, it was considered by Wieland and Scheuing (1920) to be in the nature of an addition followed by a condensation.



Fuchsin-sulphurous acid Aldehyde



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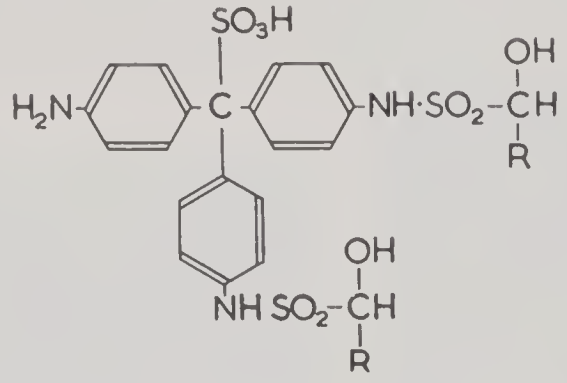

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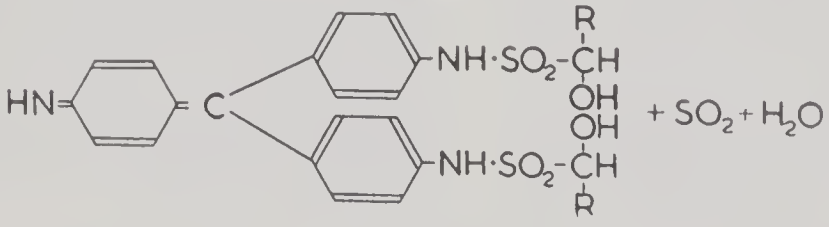
Molecular rearrangement then takes place to give :



and this compound, since it possesses the quinonoid grouping,

is coloured.

Variations in Colour of the Schiff Reaction. The possible interpretations of variation in the shade of colour developed in the Schiff

reaction have been the subject of discussion by a number of authors, some of whom have suggested that the magenta shade of fuchsin-sulphurous acid recolorized by exposure to air is the only colour given by "genuine" reactions. Others have insisted that a "genuine" reaction gives a shade considerably bluer than that of naturally recolorized fuchsin-sulphurous acid. Lison (1932) was of the opinion that wide variations in final colour occurred and that the interpretation of differences in shade should be made with caution. Gomori (1950), investigating the amine oxidase reaction of Oster and Schlossman (1942), in which the final product with Schiff's reagent was stated to be bluish in colour, determined the reaction of four different aromatic aldehydes. He noted that benzaldehyde gave a purple colour, of a similar shade to that developed in the plasmal reaction, anisaldehyde a redder shade, vanillin rose red and *p*-dimethylaminobenzaldehyde scarlet. No blue shades occurred and Gomori suggested that these might be due to alterations in the plasmal-Schiff compound regenerated from its bisulphite complex, or to the acid pH of the bisulphite. With the exception of the pH level no factor is known with certainty to be responsible for "metachromatic" effects when fuchsin-sulphurous acid is employed and I suggest that any colour whatsoever should be regarded as a positive reaction with Schiff's reagent but that departures from the usual magenta should be regarded as having unknown but possibly interesting significance.

Ammoniacal Silver Reactions

Many reducing reagents will convert ammoniacal silver solutions to metallic silver. Their reduction has been used for the demonstration of aldehydes and for certain special types of ketone, referred to below. Although ammoniacal silver solutions can be used to demonstrate the presence of plasmal, and to block the reaction of the latter with Schiff's reagent, they are most commonly employed in histochemistry to render visible the reaction products formed from glycogen and mucin by oxidation with various reagents (see methods for glycogen, Chapter IX, p. 265). According to Lhotka and Davenport (1951) they will not react with deoxyribonucleic acid previously hydrolysed by a Feulgen type of hydrolysis, and this observation, among others, caused these authors to doubt the aldehydic nature of the substance responsible for the nucleal reaction. My experience does not entirely agree with the observations of these authors, although I agree that the usual acid hydrolysis followed by short immersion in an ammoniacal silver bath gives a negative result. I have found that after hydrolysis with acids other than HCl, formic acid for instance, followed by 18 hours in the silver solution, there is an appreciable blackening of the

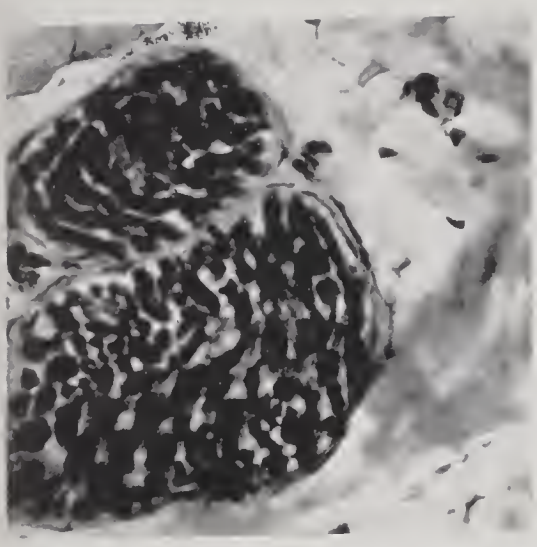


FIG. 92. Rat. Peripheral nerve. The myelin sheaths stain deep blue in this fresh frozen section. Acid Solochrome cyanine. × 360.

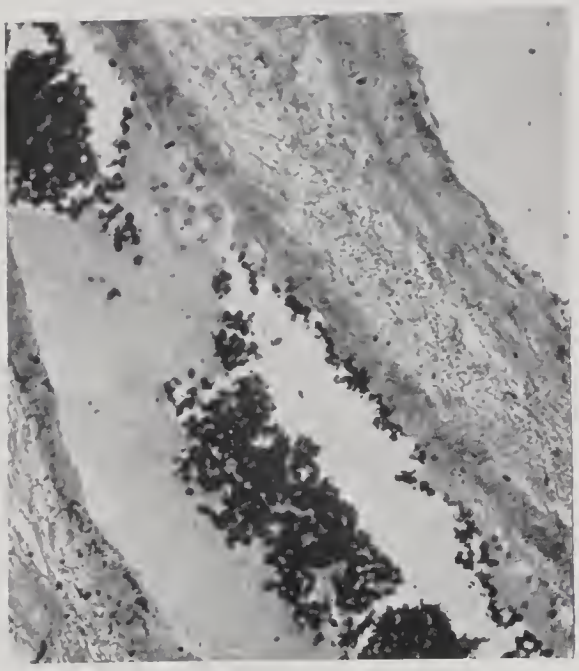


FIG. 93. Masses of black-stained red cells within a blood vessel. Performic acid—ammoniacal silver. × 100.

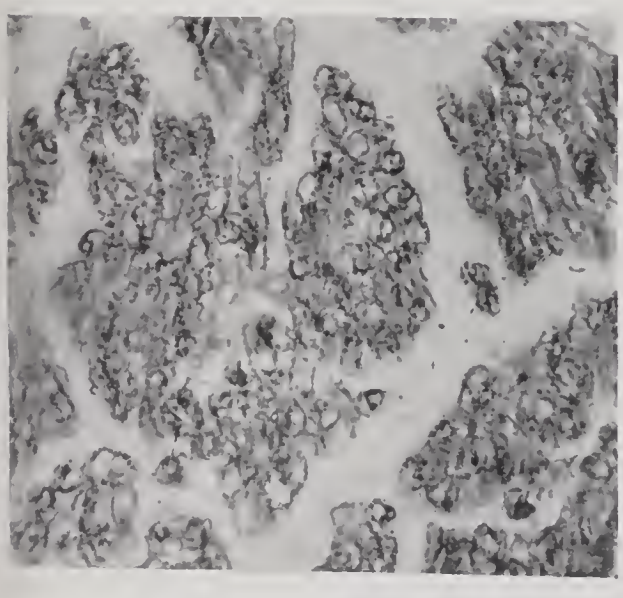


FIG. 94. Myelin sheaths stained a dark purple colour. Performic acid—naphthoic acid hydrazide—diazotized *o*-dianisidine. × 300.

FIG. 95. Human gastric mucosa, oxidized with 1 per cent. aqueous HIO_4 for 10 minutes at 22°, showing granules of mucin strongly stained. Phenylhydrazine-formazan method. × 400.

[*To face p.* 336.

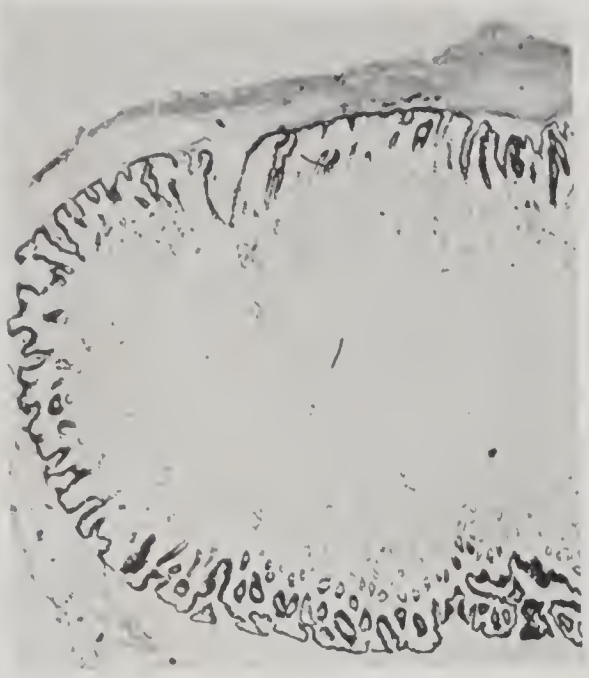


FIG. 96. Serial section to Fig. 97, identically treated except for incubation with dimedone in 5 per cent. acetic acid (60°, 2 hours) after oxidation. Schiff. × 21.

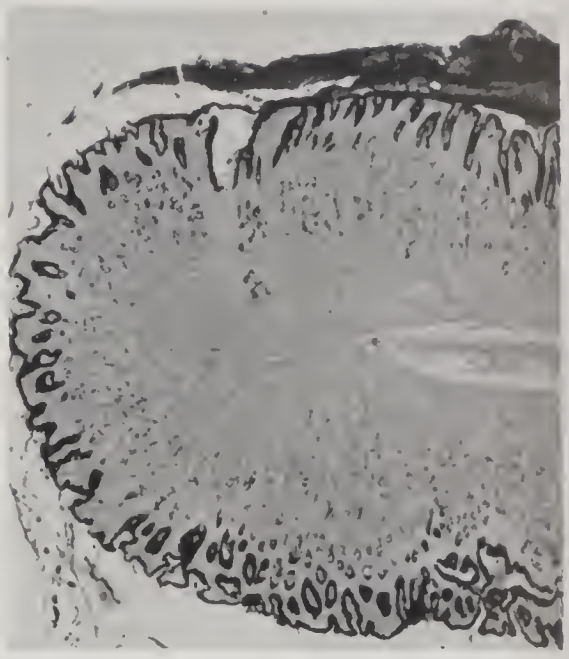


FIG. 97. Paraffin section (5·5 μ) of human gastric mucosa. Oxidized for 10 minutes at 22° with 1 per cent. aqueous HIO_4. Schiff. × 21.

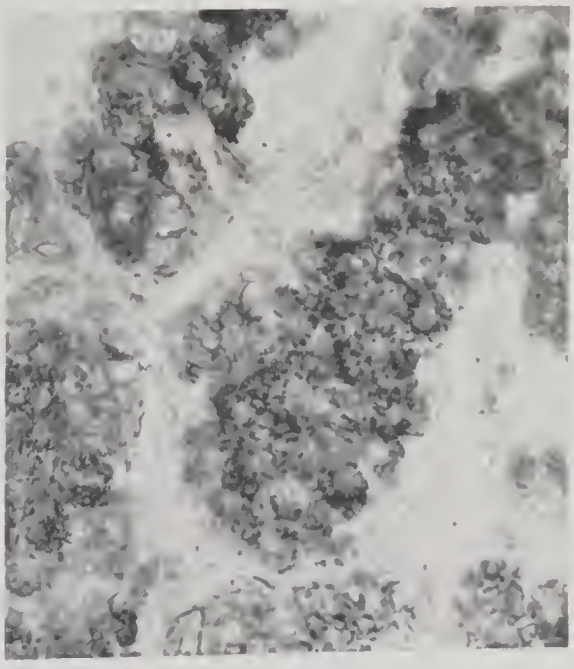
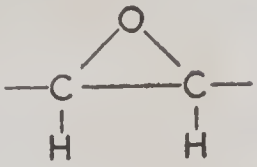


FIG. 98. Frozen section (10 μ) of human adrenal cortex, showing a strong reaction in the zona fasciculata. Naphthoic acid hydrazide—diazotized *o*-dianisidine. × 335.

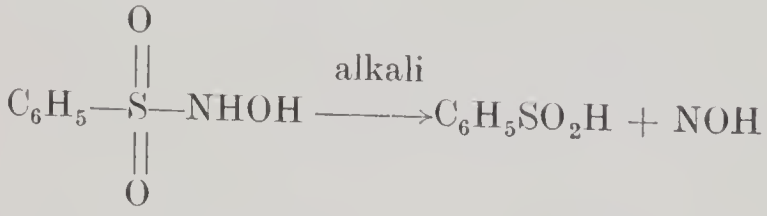
nuclei, absent from control sections. Cain (1949b) drew attention to the fact that ethylene oxide groups



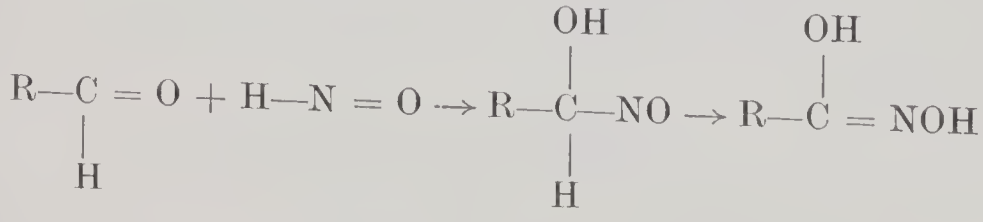
which can be formed by oxidation of fats in the presence of sulphydryls such as glutathione, will also reduce ammoniacal silver solutions. Although the presence of such groups may have to be taken into account in order to explain the silver reactions of lipid-containing materials, aldehydes remain the most likely groups to be responsible for a positive silver reaction in oxidized tissue sections.

The Nitrohydroxylamine Reaction

From time to time references to the use of this specific test for aldehydes occur in the histochemical literature. Originally suggested by Angeli (1892) and Rimini (1901), it depended on the reaction of nitrohydroxylamine with aldehydes (but not with ketones) to give substituted hydroxamic acids. The latter give an intense red colour with ferric salts. The majority of accounts refer to the use of this test with a negative histochemical result, interpreted as indicating the absence of aldehydes. Such an interpretation is certainly not valid until a positive result has been shown to occur with known tissue aldehydes and even then it is possible that potential aldehydes may fail to react. In practice, the method can be carried out with benzene sulphonyl hydroxamic acid in dilute alkali. Under these conditions the compound behaves as though it splits to form benzene sulphinic acid and an NOH group :



The latter combines with the aldehyde to form a substituted hydroxamic acid :



This gives the red colour with ferric salts. I have not succeeded in

making the method work with known aldehyde groups such as those produced by the action of periodic acid on tissue polysaccharides, and it is doubtful whether a positive histochemical reaction has ever been obtained.

Phenylhydrazine and Dinitrophenylhydrazine Reactions

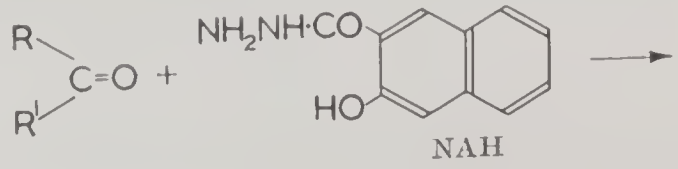
Both aldehydes and ketones condense with phenylhydrazine to form substituted hydrazones which are used extensively for chemical characterization. With aldehydes the following reaction takes place :

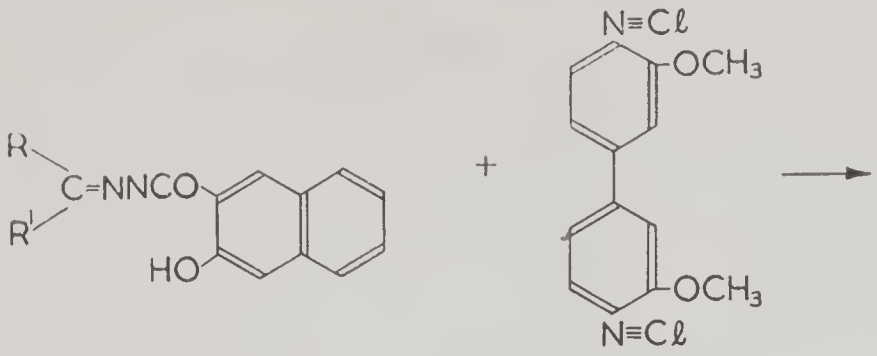
$$R.CHO + H_2N.NHC_6H_5 \rightarrow RCH = N.NHC_6H_5 + H_2O$$

Bennett (1940) was the first to make histochemical use of the phenylhydrazine reaction for demonstration as opposed to blocking, noting the yellow colour which it gave with frozen sections of the adrenal gland. He suggested that the reaction was due to ketones present in the adrenal cortex presumably in the form of fat soluble ketosteroids. Dempsey and Wislocki (1946), who used 2 : 4-dinitrophenylhydrazine for the same purpose, were also of this opinion. The colour developed by the latter reagent is stronger than that given by phenylhydrazine, but in either case it is faint and not easily visible in small structures such as chromosomes, although Danielli (1947) claimed that the dinitrophenylhydrazine reaction of hydrolysed chromosomes was identical with that given by Schiff's reagent. This author (1949) subsequently developed a substituted azobenzenephenylhydrazine sulphonic acid which gave a purple colour with aldehydes.

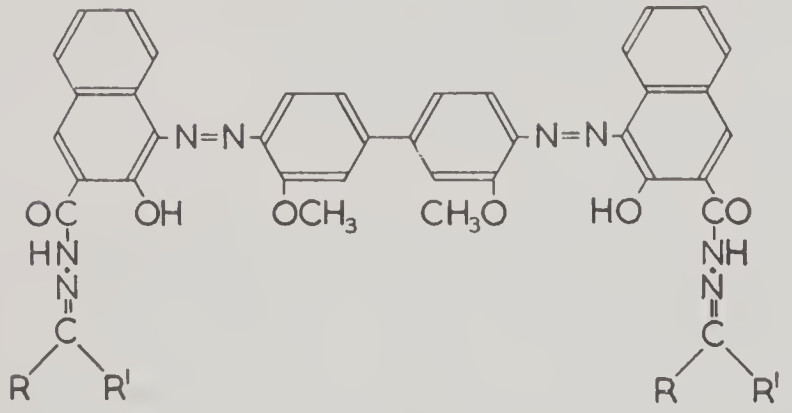
Naphthoic Acid Hydrazide Reactions

A considerable technical advance on the phenylhydrazine techniques was made independently by Camber (1949) and by Ashbel and Seligman (1949) who substituted 2-hydroxy-3-naphthoic acid hydrazide (NAH) for the various phenylhydrazides. This compound condenses with both aldehydes and ketones to give a product no more highly coloured than that given by the earlier techniques. By subsequent coupling with a diazonium salt, however, the carbonyl-hydrazide compound is readily made visible, and the authors referred to above used diazotized *o*-dianisidine for the purpose. This gives a strong bluish-purple as the final colour. The reaction is considered to take place as follows :





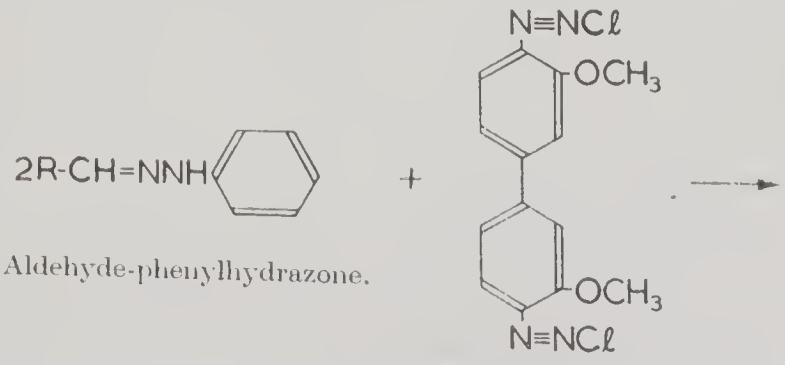
Diazotized *o*-dianisidine



Purple dye

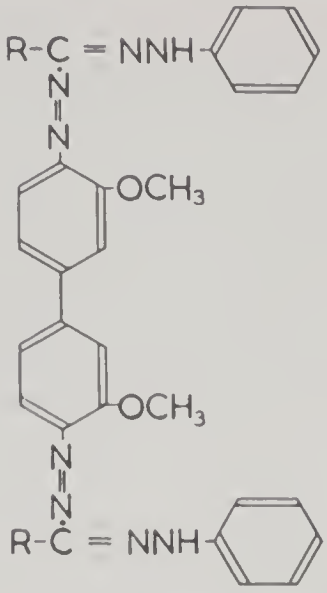
The Phenylhydrazine-formazan Reaction

This reaction was introduced by Seligman, Gofstein and Rutenberg (1949) as part of the synthesis of an iodized tetrazolium salt and it was stated by Ashbel and Seligman to be specific for aldehydes as opposed to ketones. According to these authors the phenylhydrazones of aldehydes couple with diazonium salt in pyridine to form water-insoluble, coloured, formazans. The phenylhydrazones of ketones, because of the absence of replaceable hydrogen, cannot undergo the reaction which presumably proceeds as follows :



Aldehyde-phenylhydrazone.

Diazotized *o*-dianisidine



Diformazan (purple)

The negative results obtained in formalin-fixed sections of adrenal cortex when this method was applied suggested that free aldehydes were not present. Like the original authors, I have not succeeded in making the method work with formalin-fixed adrenals, but in paraffin sections exposed to periodic acid the sites which are normally coloured by means of Schiff's reagent are coloured purplish red by the use of phenylhydrazine followed by the stable diazotate of dianisidine in watery pyridine. Fig. 95 shows granules of mucin in the gastric mucosa stained in this manner after oxidation with periodic acid. I have not so far been able to show the potential aldehydes, revealed in DNA by acid hydrolysis, by the phenylhydrazine-formazan method using a number of diazonium salts. I do not, however, consider that the negative formazan reaction in the adrenal cortex offers conclusive proof of the absence of aldehydes. Great differences in reactivity are noted between the various aldehydes in the tissues and the substances with which they combine, and the negative formazan reaction with the potential aldehyde of the Feulgen hydrolysis might well be due to protection of the hydrogen atom from substitution by the diazotate.

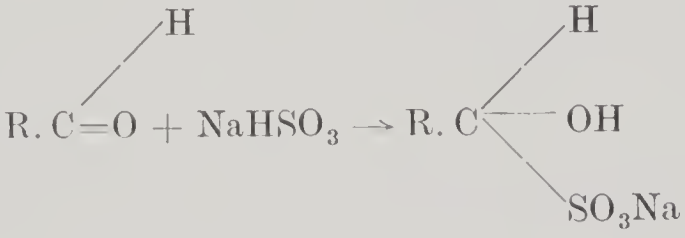
Feldman's (1950) criticism of the specificity of the NAHD reaction, on the grounds that the reagent combines with aldehydes *in vitro*, is not a valid one. Ashbel and Seligman never maintained that NAH failed to condense with aldehydes but only that free aldehydes were not present in formalin-fixed sections of adrenal cortex. Since they have not succeeded in proving this thesis in a satisfactory manner, it must be concluded that the NAHD reaction, despite certain differences between its results and those obtained with leucofuchsin, is a measure of tissue aldehydes produced partly by hydrolysis of acetals and partly by oxidation of fatty acids.

Blocking Reactions for Aldehydes

Aldehydes are exceedingly reactive compounds and it is not surprising that a very large number of methods exist for their chemical characterization. What is surprising, however, is the fact that so many have been successfully applied to histochemistry. Nearly all those which have been used in this way are given below, approximately in historical order, and judgment as to which of them is preferable for any given purpose may usefully be withheld until all have been considered.

Bisulphite

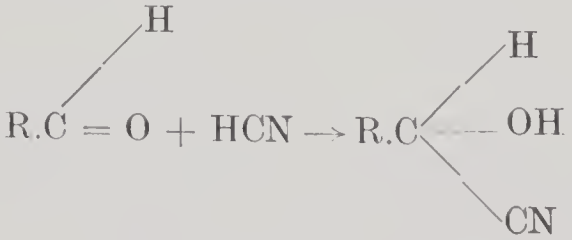
Sodium bisulphite forms addition compounds with aldehydes and ketones of the type



which can be broken down to aldehydes or ketones by the addition of dilute acids and alkalis. The bisulphite method was used by Feulgen and Voit (1924) to block the reaction of plasmal with Schiff's reagent.

Cyanides

The abolition of the plasmal reaction by cyanides, noted by Gérard (1935), was attributed to their poisoning action on the oxidant enzymes which he considered responsible for the reaction, in tissues other than myelin. It is more probable that traces of HCN, present in solutions of sodium or potassium cyanide, form addition compounds with aldehydes or ketones in the tissues.



Cyanohydrin

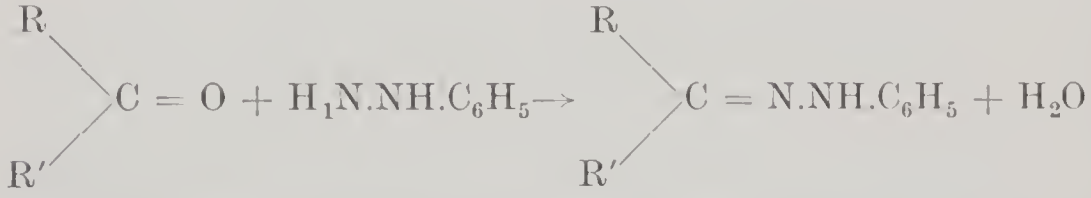
Cyanohydrins readily hydrolyse to α-hydroxy carboxylic acids.

This type of reaction was particularly favoured by Gomori (1953) who recommended the use of KCN in phosphate buffer at an acid pH. Lillie (1956) noted that aldehydes blocked with cyanide would react again as aldehydes after oxidation with periodic acid. This means that

cyanohydrins, or more probably α-hydroxy carboxylic acids, can be attacked by periodic acid as well as by lead tetraacetate.

Phenylhydrazines

The reactions of primary amines with aldehydes have long been known. Verne (1929b) noted that treatment with phenylhydrazine would reverse the plasmal reaction in the adrenal cortex and medulla and Bennett (1939, 1940) made particular use of the reaction in his studies of the adrenal cortex of the cat. The histochemical use of the phenylhydrazine reaction is now often known by his name, although this usage, if sanctioned at all, should be reserved for the colour reaction rather than for the blocking reaction. According to Wild (1947), monosubstituted and unsymmetrically disubstituted hydrazines condense with the carbonyl groups of both aldehydes and ketones to form substituted hydrazones.

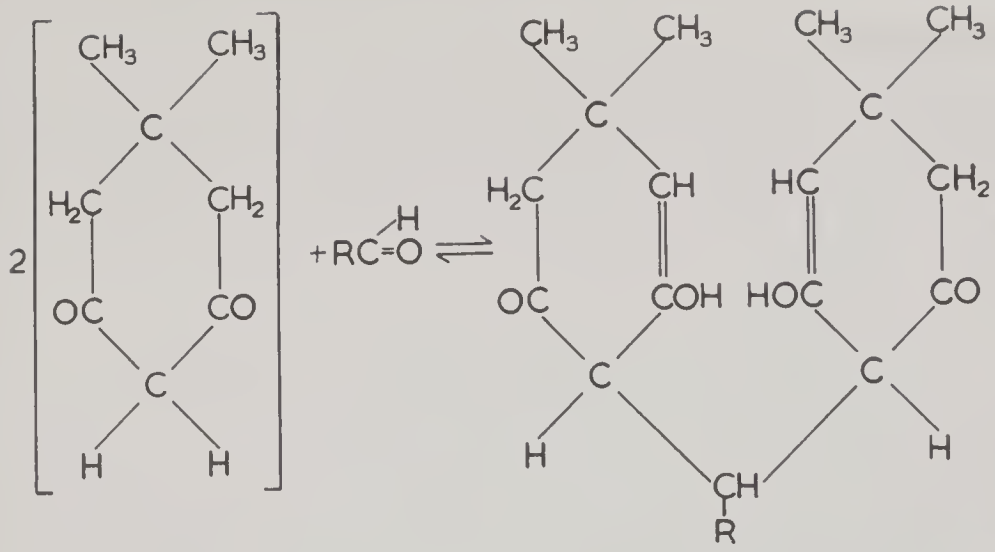


Phenylhydrazine quickly forms these derivatives, which are yellow in colour, and a reaction may be obtained in tissue sections in a few hours. Danielli (1949) used 2 : 4-dinitrophenylhydrazine as a blocking reagent for the plasmal reaction and Lhotka and Davenport (1951) used *p*-nitrophenylhydrazine in their study of the Feulgen technique. Naphthoic acid hydrazide, and no doubt very many other hydrazides, can be used in a similar manner. Phenylhydrazine blockade is resistant to further oxidation by periodic acid.

Dimedone

This agent (5 : 5-dimethyl-*cyclo*-hexane-1 : 3-dione) forms condensation products with aqueous, alcoholic or glacial acetic solutions of aldehydes.

If acetic acid solutions are employed the internal anhydride of this product is formed. The dimedone reaction was used by Verne in his extensive studies on plasmal and was presumed by Cain (1949b) to react with ketones as well as with aldehydes. Wild (1947), however, states that no condensation occurs with ketones and that the reaction is specific for aldehydes. If it is employed at room temperature the dimedone reaction is slow. Fig. 96 shows the effect of dimedone (60° for 2 hours) on a section of gastric mucosa oxidized for 10 minutes with 1 per cent. aqueous periodic acid. Fig. 97 shows the untreated control section. The abolition of the reaction with Schiff's reagent

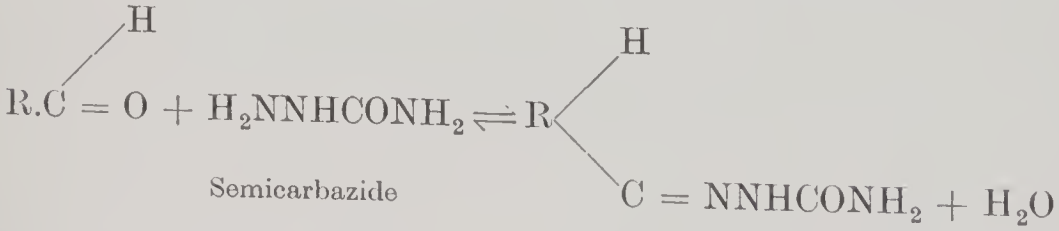


is rapid in the connective tissues and muscle (except for glycogen and mast cell granules) and fairly rapid in free mucin in the lumen. In this short period dimedone has no effect on the staining intensity of the mucinogen granules of the mucosa. Prolonged treatment, for 24 hours at 60°, still fails to abolish the positive reaction in the latter situation. The significance of these observations remains to be explained, though it is probable that they reflect both a quantitative difference in the number of aldehyde groups in a given structure and the degree of protection from dimedone caused by stereochemical factors.

Dimedone blocking is stable to further oxidation with periodic or peracetic acids.

Semicarbazides and Thiosemicarbazides

These very reactive compounds form insoluble products (semicarbazones, thiosemicarbazones) with aldehydes and ketones. The reactions are reversible and the rate at which they proceed depends on the hydrogen ion concentration. Since semicarbazides are basic substances an acid pH favours the formation of semicarbazones



Semicarbazide

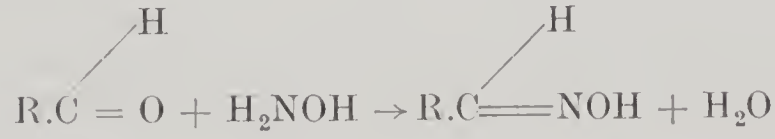
In much of the early research on plasmal, semicarbazides were used to block the Schiff reaction of tissue aldehydes; Bennett (1940) used a 10 per cent. solution in absolute methanol, with the addition of sodium acetate, but the method given in Appendix 12 using acetate buffer at pH 4·5, is preferable. Thiosemicarbazides may be used in a similar manner, but they have no advantage in the simple blocking reaction.

Sulphanilic Acid and Sulphonamide

A further example of the amine-aldehyde condensation, already illustrated in the semicarbazide and phenylhydrazine reactions, is given by the technique of Oster and Mulinos (1944), who used sulphanilic acid and sulphonamide as blocking reagents in their studies of tissue aldehydes. Boscott and Mandl (1949) used this type of reaction on sections of rat adrenal cortex previously treated with $HgCl_2$. They found, subsequently, that no recolorization of Schiff's solution occurred. As mentioned earlier in this chapter, they were unable to effect condensation *in vitro* with the two ketosteroids which they tried, but in spite of this evidence it is certain that carbonyl groups other than aldehydes will condense with sulphanilic acid or sulphonamide although the rate of reaction may be slow.

Hydroxylamine

Yet another example of an amine-aldehyde condensation is given by the reaction with hydroxylamine which occurs readily with both aldehydes and ketones. The condensation products are known as aldoximes or ketoximes.



The rate of formation of these derivatives is dependent on the hydrogen ion concentration, with an optimum at pH 4·7, decreasing on either side of this point but increasing again in strongly alkaline solutions (Ölander, 1927). In practice hydroxylamine is used in the presence of excess sodium carbonate or acetate. The reaction was used by Danielli (1949) to block free tissue aldehydes prior to the release of acetal aldehydes by treatment with either $HgCl_2$ or N-HCl.

Staple (1958) observed that blocking of aldehydes with hydroxylamine was reversed by oxidation with periodic acid. It cannot therefore be used to block existing aldehydes in frozen sections, for instance, before carrying out the PAS reaction.

Aniline Chloride and Aniline

Lillie (1954) recommended the use of this reagent to produce aldehyde blockade with relatively short incubation (15 minutes). The blockade so produced is stable to periodic acid oxidation but some tissues require much longer than the minimal incubation time. Lillie and Glenner (1957), testing aniline blockade of various carbonyl groups, found that the reaction was more rapid and complete when carried out with a molar solution of aniline in acetic acid.

Cannizzaro Reactions

Aldehydes undergo a peculiar change when treated with alkali. An intermolecular displacement of oxygen occurs, one molecule of aldehyde being reduced at the expense of another which is oxidized to an acid (cf. Karrer, 1950).

$$2R.C(=O)H + NaOH \rightarrow RCH_2OH + RCOONa$$

This reaction was discovered by Cannizzaro and now bears his name. Lhotka and Davenport (1951) made use of the method in a modified form which they called a crossed Cannizzaro reaction. They considered that the supposed aldehyde radicals produced in deoxyribonucleic acid by Feulgen hydrolysis would have only limited motion and could not, therefore, be expected to react freely with each other. Additional aldehyde groups in the form of formaldehyde were therefore provided and this crossed Cannizzaro reaction was found to prevent the recolorization of Schiff's solution by the nuclei in hydrolysed blocks of tissue ; sections were not used since these could not withstand the prolonged action of strong alkali.

Meervein-Ponndorf Reaction

This reaction was also used by Lhotka and Davenport (1951), in the studies already considered above. It depends on the addition of hydrogen to the carbonyl group, the product being a primary alcohol, and may be written as follows :

$$R.C(=O)H + H_2 \rightarrow RCH_2OH$$

In practice the reaction is more complicated and several views apparently exist as to the mechanism involved. Lhotka and Davenport used blocks of tissue, exposed to Feulgen hydrolysis, and refluxed these for 2–4 hours in *iso*propyl alcohol to which excess aluminium ethoxide was added. According to Karrer (1950) the reaction with aluminium alcoholates proceeds as follows :

$$R.C(=O)H + Al(OCH_2.CH_3)_3 \rightleftharpoons RCH_2OAl(OCH_2CH_3)_2 + CH_3CHO$$

and this intermediate compound reacts with water :

$$RCH_2OAl(OCH_2CH_3)_2 + 3H_2O \rightarrow RCH_2OH + Al(OH)_3 + 2CH_3CH_2OH$$

An alternative explanation for the reaction, derived from Gilman (1943) has been given in Chapter VIII, p. 200. By this means Lhotka and Davenport were able to block the Feulgen reaction in previously hydrolysed tissues. They were unable to draw any firm conclusions as to the nature of the tissue group involved. The Meervein-Ponndorf reduction cannot, in any case, be used to distinguish between aldehydes and ketones since both are able to react.

Uses of the Various Staining and Blocking Techniques for Aldehydes

There are two chief histochemical uses of the above techniques: (1) For selective blocking of aldehydes, i.e. to block free aldehydes prior to the plasmal reaction or to block aldehydes and leave ketones free to react. (2) For confirmation that a tissue group giving a positive Schiff reaction, by whatever means it may have been produced, is aldehyde rather than ketone, peroxide, hydroperoxide, ethylene oxide or some other group.

The efficiency of the blocking reagents can be assessed by their effect in preventing the recolorization of Schiff's solution by tissue aldehydes, and these can conveniently be produced by periodic acid oxidation. It must be remembered, however, that the configuration of the group produced in the tissues will have a pronounced effect on the rate of condensation with the various reagents and that the reagent, say, which condenses best with the aldehydes produced from glycogen by periodic acid will not necessarily be the best reagent to block the groups produced by treatment of acetals with $HgCl_2$. All the reactions are known to proceed faster with aldehydes than with ketones and with the aliphatic than with the aromatic series.

Leaving theoretical considerations aside, for selective blocking of tissue aldehydes six of the methods discussed are of practical utility. These are the bisulphite, hydroxylamine, semicarbazide, phenylhydrazine, aniline chloride and sulphanilic acid reactions. With hydroxylamine in sodium acetate, at room temperature, condensation with tissue aldehydes of all varieties is rapid and apparently complete, but the product is susceptible to further oxidation. The semicarbazide and phenylhydrazine reactions act similarly but are considerably slower. Their products are resistant to oxidation. The best method for routine use is probably Lillie's aniline chloride.

For the accurate characterization of a tissue component as aldehyde, only three reactions out of the fourteen staining and blocking reactions listed have any claim to specificity. The first, which gives a coloured product, is the nitrohydroxylamine reaction of Angeli and Rimini. This reaction is not of practical use in histochemistry. The second, which also gives a coloured product,

is the phenylhydrazine-formazan reaction and the third, used solely for blocking, is the dimedone reaction. In respect of the second, I consider that if a positive result is achieved it may be concluded that the tissue group under investigation is an aldehyde, while a negative result cannot be said to exclude this possibility. This is because the reaction is not technically easy and because in a given section it tends to show only regions giving a strongly positive Schiff reaction. Weaker sites are often negative. There is also a strong probability that the configuration of the tissue aldehyde, even if it allows condensation with phenylhydrazine, may not permit the hydrogen substitution necessary to achieve formazan production. The dimedone reaction, though theoretically specific for aldehydes, is sometimes found to block these exceedingly slowly in practice. It is often difficult to distinguish the reduction in recolorization of fuchsin-sulphurous acid caused by dimedone from that observed in control sections treated with the solvent (acetic-alcohol) only. The failure of dimedone to block the reaction of a given component does not therefore mean that this is not due to aldehyde.

Much more use may be made of all these reactions in the future and comparative studies should be made of their various effects. The rapid blocking by dimedone, for example, of the " depolymerized " gastric mucin of the lumen, compared with its exceedingly slow blocking of the " polymerized " product in the cell, requires further investigation and explanation. The difference between hydroxylamine and phenylhydrazine blocking on secondary tissue components (say, glycogen or mast-cell granules), when the Schiff reaction of the primary component (say, periodic-acid-oxidized mucin) has been reduced equally by both, is another example requiring further elucidation.

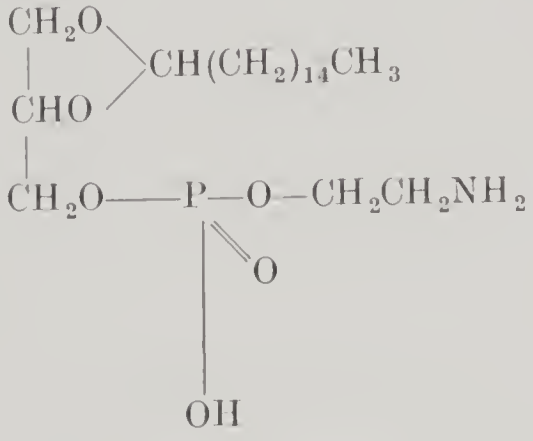
The Plasmal Reaction

Chemistry of the Reaction

The acetal phosphatides, for which this reaction is specific, are widely distributed in animal tissues where they commonly occur in association with ester phosphatides. In such combinations they may be present in amounts up to 12 per cent. Attention was first drawn to their existence by Feulgen and Rossenbeck (1924), who noticed, in performing the nucleal reaction (Chapter VIII) now known by the first author's name, that substances occurred in the cytoplasm which gave a reaction with Schiff's reagent in sections not exposed to the hydrolysing action of N-HCl. Feulgen and Voit (1924) subsequently showed that the reaction with Schiff's solution could be abolished by previous treatment with sodium bisulphite or phenylhydrazine (see section on

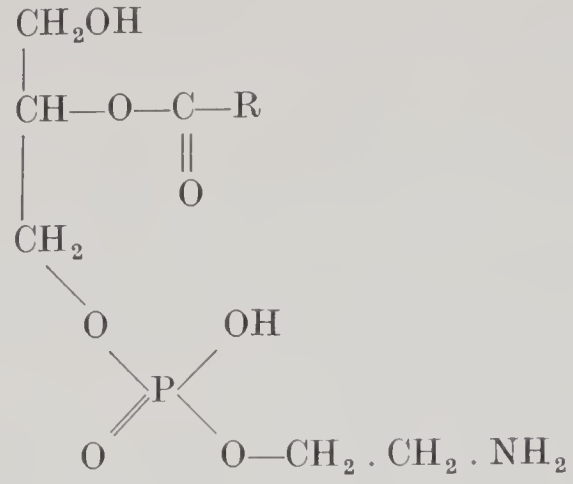
aldehyde-blocking techniques), and they concluded that the reacting groups were aldehydes. These cytoplasmic aldehydes they called plasmals. Using frozen sections, Feulgen and Voit demonstrated that the plasmal reaction could usually be abolished by the application of lipid solvents, but some of the lipids which gave rise to the reaction were sufficiently strongly attached in the tissues to resist the process of paraffin embedding. They noted that the plasmal reaction could be intensified by brief treatment of the tissues with mercuric chloride or by acid hydrolysis, and that the acid Schiff's reagent could effect a similar hydrolysis. Previous treatment with alcohol, if sufficiently prolonged, reversed the positive reaction obtained by the use of $HgCl_2$. In view of these findings, Feulgen and Voit considered that the aldehyde plasmal was derived from a lipid precursor and this precursor they named plasmalogen.

Feulgen and Behrens (1928) succeeded in isolating plasmalogen from the phospholipid fraction in which it occurred by saponifying the latter with alkali, and the same authors (1938) were able to oxidize plasmalogen to stearic and palmitic acids. Later Feulgen and Bersin (1939) recognized that the fatty aldehyde of plasmalogen was joined to glycerol by an acetal linkage and that the whole structure was that of an acetal phosphatide :



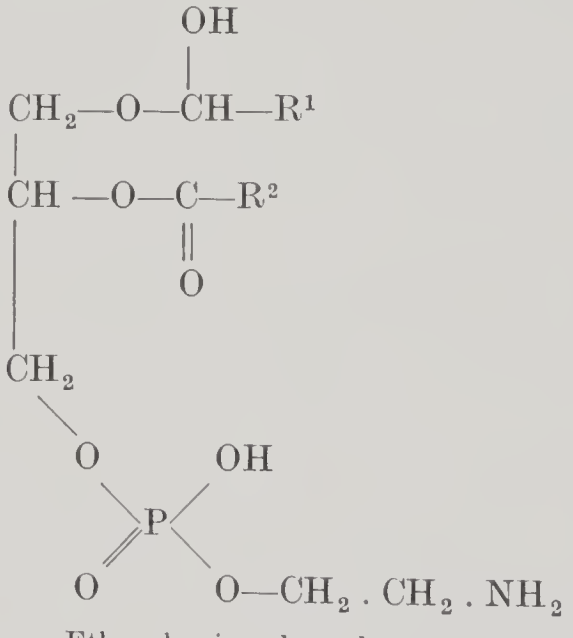
α-palmital phosphatido

Note the relationship of this structure to that of α-kephalin (p. 291). More recent work by Klenk and Böhm (1951) and by Klenk and Debuch (1954, 1955), which is described in English by Debuch (1957), has added to our knowledge of the basic chemistry of the plasmalogens. These authors noted that in the case of brain tissue it was impossible to separate acetal phosphatide from ethanolamine although the former is insoluble and the latter soluble in ether. They obtained from an acid-treated fraction of brain lipids, in addition to phosphatidyl ethanolamine (formula on p. 291), an ethanolamine-containing lysokephalin whose structure is given below:



Ethanolamine-lysokephalin

Since it was produced by acid treatment this component could only have come from the original acetal phosphatide of the brain by splitting off of an aldehyde group. The formula of ethanolamine plasmalogen is therefore :



Ethanolamine plasmalogen

Klenk and his co-workers found that two-thirds of the ethanolamine kephalin fraction of brain, obtained by Folch's method, consisted of acetal phosphatide. Stammler *et al.* (1954) have shown that the plasmalogen content of the myelin sheaths is twice that of the cerebral cortex, and in this case 55 per cent. of the ethanolamine kephalin fraction consists of the plasmalogen. Choline-containing plasmalogens have not been found in brain but Lovern *et al.* (1957) found that 55–60 per cent. of the lipids of ram spermatozoa were choline-containing plasmalogens. In beef heart lipids also, the plasmalogens are of the choline-containing type (Klenk and Debuch ; 1954, 1955).

The action of $HgCl_2$ upon the acetal linkage is of fundamental importance and the conclusions of Feulgen and his school, which are clear and simple, had best be stated at this point, before complications and contradictions due to later histochemical enquiry by different authors are introduced. According to Feulgen, acetal phosphatides, which on their own account do not react with Schiff's solution, are rapidly split by $HgCl_2$ to yield palmital, stearal and higher aldehydes, which react as such and recolorize leucofuchsin.

Histochemistry of the Reaction

The literature referring to the plasmal reaction was thoroughly reviewed by Cain (1949b), who considered the most important histochemical investigations into its nature, especially those of Lison, Verne and Gérard. Cain attributed the considerable measure of disagreement achieved by these authors partly to variations in the techniques employed. As will be seen later in this chapter, both Cain (1949a) and Hayes (1949) have wisely redefined the plasmal reaction in terms of a specific technique.

The prolonged investigations of Verne upon the subject of the plasmal reaction extended from 1928 to 1942 (Verne, 1928a, b and c, 1929a and b, 1936a and b, 1937, 1940 ; Verne and Verne-Soubiran, 1939, 1942). He observed (1928c) that various lipid structures could be made to give a positive plasmal reaction by oxidation with $KMnO_4$, CrO_3 or H_2O_2, and he considered that the groups responsible for the reaction were aldehydes produced by oxidation of hydroxyl groups in the phospholipin molecule, in other words that the aldehydes were derived from alcohols. Following on this reasoning Verne made the assumption that substances in the tissues which give a positive plasmal reaction without oxidative procedures must already exist in an oxidized state. In the same paper Verne also noted that prolonged oxidation with the reagents described would reverse the reaction of structures initially plasmal positive. Later (1929a), as the result of studies on the oxidation of pure fats, he decided that the aldehydes produced by oxidation were not formed from alcohols but from unsaturated (double) bonds. When they were derived from natural oxidative processes, the aldehyde groups were thought to exist in some loose combination in the tissues, which could be revealed by relatively mild procedures such as treatment with $HgCl_2$, for instance. Verne (1937) also showed that formaldehyde fixation prevented certain tissue lipids from giving a plasmal reaction in the untreated state, but that they could be made to react once more by means of $HgCl_2$.

Gérard (1935) studied the distribution of plasmal in various tissues and was the first to show that formol-fixed material could be used

for such a study provided that thorough washing followed the action of the fixative. Unfortunately for our relatively simple conception of the plasmal reaction Gérard also studied the distribution of what he called " oxidases " by means of the " Nadi " reaction (Chapter XVII) and found complete parallelism between the sites of plasmal and those containing " oxidase." Lison (1936a) confirmed Gérard's observations, but in addition he found that the positive " Nadi " reaction given by fats was not inhibited by cyanide and that the substance concerned would give a positive reaction with benzidine in the presence of a peroxidase. Lison therefore considered the " Nadi "-positive, benzidine-positive material to be a peroxide. Verne had concluded that the " Nadi " reaction recorded the process of autoxidation (peroxidation) of fats, at a time when it was actively proceeding, and that the plasmal reaction recorded the products of autoxidation. Later still (1940), however, he recorded his opinion that the " Nadi " reaction showed the presence of a secondarily acquired phenol oxidase system.

Oxidation of Fats. The process of oxidation of unsaturated fatty acids is now thought to proceed by the formation of a hydroperoxide (OOH) group on the carbon atom adjacent to the double bond, followed by the opening of this bond to form keto and hydroxy groups and, subsequently, by breaking of the carbon chain to form aldehydes. All the above groups may co-exist in any particular fatty acid undergoing oxidation. Gomori (1942) dealt with the action of formalin on tissue lipids which he considered to be in the nature of an oxidation. He stressed the necessity of keeping tissues designed for the study of the plasmal reaction from contact with air or oxygenated water.

Some additional evidence for this explanation of Gérard's results, namely that they depend on the presence of a peroxide stage in the oxidation of fats, may be found in the following, at first sight unrelated, observations. Dam and Granados (1945a) demonstrated that the formation of peroxides preceded, and in some instances paralleled the production of a yellow-brown pigment in the adipose tissue of rats on a high cod-liver oil, low vitamin E, diet. They also (1945b) showed that the peroxidation effect of cod-liver oil was due to its highly unsaturated fatty acids. Glavind *et al.* (1949) developed a histochemical method for demonstrating these fat peroxides, using leuco-dichlorphenolindophenol with hæmin as catalyst, which gave a red colour at the sites of peroxidation, and using this method Granados and Dam (1950) found that in the early stages of brown pigment formation the parallelism previously observed chemically could be shown to exist histochemically (see Chapter XXIII, p. 663 and Appendix 23). In later stages, however, a lack of parallelism was observed and these authors concluded that the insoluble yellow-brown pigment

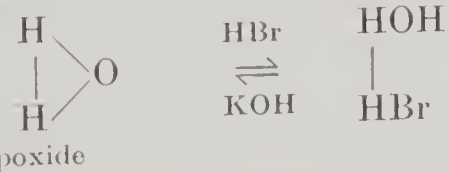
represented an abnormal product formed from highly unsaturated fatty acids beyond the fat-soluble, yellowish, peroxide stage. Beyond showing that the yellow-brown pigment was acid fast, no additional histochemical studies of its nature were attempted. The views of the various authors referred to above are summarized in Table 24.

TABLE 24

Histochemical Views on Fat Oxidation

Author	Reacting substance	Mechanism	Product	Demonstrating reaction
Feulgen and Voit (1924) .	Acetal-phosphatides	$HgCl_2$	Aldehydes	Schiff
Verne (1940) .	Unsaturated fatty acids	Oxidizing enzyme	Aldehydes	" Nadi " and Schiff
Gérard (1935) .	Unsaturated fatty acids	Atmospheric O_2	" Oxidizer "	" Nadi "
Dam and Granados (1945a).	Unsaturated fatty acids	Atmospheric O_2	Peroxide	Leuco-indophenol
Lison (1936) .	Unsaturated fatty acids	Atmospheric O_2	Peroxide	" Nadi " and Benzidine

Recent work by Lea (1956) has indicated that the best naturally occurring antioxidant is vitamin E ; other compounds having this property are the dihydrocaffeic acids, fluorones, tannins and catechol derivatives. Many acids, themselves possessing no intrinsic anti-oxidative property, considerably increase the anti-oxidative effects of phenolic substances. Among these acids are citric and phosphoric acids and ascorbic acid and kephalin. Davidow and Radomski (1953) regard the oxidation of unsaturated compounds as a detoxication process. They suggest that the reactive C = C bond is first oxidized to an epoxide and this is then hydrolysed to a diol. Anhydrous HBr in dioxane converts epoxides into halohydrins and these can be hydrolysed back to epoxides with KOH.



Epoxide

This reaction has not been used histochemically. The whole subject of the autoxidation of fats and related substances has been reviewed very ably by Holman (1954), whose article should be consulted for further details.

Criticisms of the Plasmal Reaction. In view of more modern experience in the oxidation of fats, criticism can be levelled at the method of applying the plasmal reaction employed by Feulgen and his collaborators. They used unfixed sections and attached these to the slides

by heating, a process which might well lead to initial oxidation especially when subsequently reinforced by the action of $HgCl_2$. Feulgen particularly stressed the necessity for examining control sections unexposed to $HgCl_2$ and the techniques of Verne may be criticized on the grounds that no control sections were employed and also because he occasionally used $HgCl_2$ as a fixative, which precluded the use of controls. Danielli (1949), in his critical study of the plasmal reaction, raised and answered a number of questions. Using three additional techniques for localizing aldehydes (ammoniacal silver, phenylhydrazine and a substituted phenylhydrazide) he found that with each of these the distribution of plasmal was the same as with Schiff's reagent. He concluded that these additional reactions confirmed the specificity of the plasmal reaction for tissue aldehydes. The possibility of diffusion of the aldehyde-Schiff compound was investigated and rejected. Cain (1949b) criticized the plasmal techniques used by Danielli because an acid fixative was used (acetals were shown by Feulgen and Bersin to be destroyed in acid media), and because of a supposed lack of control sections. Danielli (1950) replied to these criticisms with a restatement of his method of distinguishing free aldehydes from acetals and from aldehydes produced by oxidation. He apparently regarded Schiff-positive groups produced by $HgCl_2$ as aldehydes produced by oxidation. His final techniques were as follows :

After neutral formalin fixation: (1) Expose sections to leucofuchsin without HCl or $HgCl_2$ pretreatment. This demonstrates free aldehydes only. (2) Use cold N/10 HCl prior to leucofuchsin. This shows both *free aldehydes and acetals* (treatment with hydroxylamine before exposure to acid reveals only acetals). (3) Cold N/10 HCl followed by hydroxylamine then by $HgCl_2$ shows only *aldehyde due to oxidation.*

It is probable that most workers would disagree with Danielli's conception of the role of $HgCl_2$.

The True Plasmal Reaction. After some careful experimental work Cain (1949a) drew a distinction between the true Feulgen-plasmal reaction and the Feulgen-Verne or pseudoplasmal reaction, considering Danielli's plasmal to belong to the second variety. According to his definition the true plasmal reaction, which is due to the release of higher fatty aldehydes from acetal phosphatides by the action of $HgCl_2$, is progressively reduced and finally destroyed by prolonged fixation. The Feulgen-Verne or pseudoplasmal reaction, on the other hand, increases in intensity with the duration of fixation provided oxygen is not excluded. Mercuric chloride does not influence the pseudoplasmal reaction. The investigations of Waelsch (1950) show that caution is necessary in evaluating the higher fatty aldehydes detected by Schiff's reagent. Both the age of the preparation and the accessibility of the lipids influence the amounts detected *in vitro.* After

five crystallizations as the cadmium complex Waelsch found that a sample of lecithin, stored for 4 days over sodium hydroxide at 15 mm./Hg. gave a fuchsin colour value equal to 70 γ palmitaldehyde. After 8 days the value was 185 γ and after 14 days 235 γ per 100 mg. These figures he attributed to the progressive oxidation of lecithin to higher fatty aldehydes. The evidence produced by Verne to show that aldehydes were responsible for his reaction was based on the assumptions that all positive plasmal reactions were due to a single component and that the techniques which he used for revealing and for blocking the aldehydes (see below) were specific for these compounds. According to Cain such assumptions are unjustified.

Modern Views on the Plasmal Reaction. The results obtained by Hayes (1947, 1949) substantially agree with those of Cain. Hayes maintained that prolonged fixation in formalin would oxidize all acetal groups and unmask Schiff-positive material which was unaffected by $HgCl_2$ in control sections. This is certainly true in the case of the adrenal cortex with which he was mainly concerned. He regarded the reaction secondarily developed by formalin fixation as demonstrating aldehyde-containing lipids other than acetals, whereas Cain believed that groups other than aldehyde, also produced by autoxidation, were responsible. Hack (1952) made a very complete study of the distribution of plasmals in animal tissues, using freeze-dried material embedded in carbowax.

Hayes (1949) redefined the plasmal reaction and insisted on the following three points in his technique for its demonstration :

(1) Brief fixation in 10 per cent. formalin.
(2) Brief action of $HgCl_2$ on frozen sections.
(3) Negative control sections from the same block.

(Between stages 1 and 2, I would add brief treatment with hydroxylamine.) All material reacting positively in control sections could not, according to Hayes, be regarded as derived from acetal lipids. Belt and Hayes (1954) made a critical evaluation of procedures for carrying out the plasmal reaction which should be consulted by those interested in applying the reaction. In Appendix 12 the methods of Cain and Hayes are both given, but if freeze-drying is available, Hack's method is preferable.

Present Status of the Reaction. To most histologists the plasmal reaction is of little importance in itself, but it is an important complication in the interpretation of other reactions involving Schiff's reagent. The established position of the Feulgen nucleal reaction and the growing importance of the periodic acid-Schiff technique therefore necessitate a thorough understanding of the problems involved and of the nature of substances, outside the main ones with which either technique is concerned, which may give positive reactions with Schiff's

solution. As a diagnostic procedure in histochemistry the true plasmal reaction may be of value in suggesting or confirming the presence of ethanolamine or choline-containing phospholipids in any particular structure, provided that neutral fats can be shown to be absent. Since the detection of these phospholipids by histochemical means, when they are present in small quantities, is made difficult by the insensitivity of the methods available, we can take advantage of the sensitivity of the true plasmal reaction to make a presumptive diagnosis. In the last 10 years interest in the plasmal reaction has centred largely upon the question whether some of the reactions used for demonstration of ketosteroids are not due entirely to the presence of plasmalogens. This particularly concerns the Bennett (phenylhydrazine) and Ashbel-Seligman (naphthoic acid hydrazide) reactions whose chemistry and specificity are considered in the section with follows.

Reactions of Aldehyde Reagents with Ketones

In the foregoing account of the various aldehyde reactions used in histochemistry references to ketones were purposely kept to a minimum to avoid confusion. Many of the reactions dealt with, however, are equally positive with ketones *in vitro* and a complication in interpretation is thus introduced. This has largely been resolved by the work of Karnovsky and Deane (1955) which brings to an end the protracted arguments of the past decade. This work, which is considered at the end of this chapter, makes much of the intervening discussion on the reactivity of the various reagents wholly academic.

Schiff's Reagent

Many groups other than aldehydes are known to recolorize leucofuchsin, but the most important ones are either oxidizers or α-ketones. Dempsey and Bassett (1943) and Dempsey and Wislocki (1944, 1946) claimed that α-hydroxyketones (α-ketols) such as deoxycorticosterone would recolorize Schiff's solution after treatment with $HgCl_2$. They supposed that this treatment would convert the ketone into an aldehyde. Boscott *et al.* (1948) performed the true plasmal reaction on samples of free deoxycorticosterone but could obtain no evidence of oxidation to an aldehyde. They considered that typical hydroxyketones would not react as Dempsey had suggested but that atypical α-ketols might possibly do so. Albert and Leblond (1946), Oster and Oster (1946), Ashbel and Seligman (1949) and Nicander (1951) also failed to achieve the plasmal reaction with ketosteroids. Karrer (1950) agrees that Schiff's reagent itself would react with certain easily oxidized α-ketols but not with the majority of ketones. In histochemistry, therefore, the presence of ketones is unlikely to be responsible for a positive Schiff reaction.

Ammoniacal Silver Reagents

Many reducing reagents will convert ammoniacal silver solutions to metallic silver. Their reduction has been used for the demonstration of aldehydes and certain special types of ketones, particularly those with a carbonyl group at C^{20} and an adjacent hydroxyl at C^{21}. Both Bennett (1940) and Reichstein and Shoppee (1943) were of the opinion that the reaction could demonstrate the presence of ketosteroids in tissue sections, yet proof that aldehydes were not responsible is lacking. It is not permissible to use ammoniacal silver reagents to demonstrate ketones histochemically, since their specificity for this purpose is altogether too low.

Phenylhydrazine Reagents

The specificity of the Bennett and similar reactions for ketosteroids was questioned by Gomori (1942) who pointed out that the reaction was certainly given by tissue aldehydes. Later (1950) he concluded that a positive phenylhydrazine reaction signified only an overwhelming bulk of plasmal, and later still (1953) that hydrazines absolutely did not react with ketones *under histochemical conditions*. This conclusion has not been refuted although there is plenty of evidence that steroids and hydrazides react *in vitro*. Progesterone, for instance, reacts at C3 and C20 with two molecules of hydrazides. The work of Albert and Leblond (1946), Claesson and Hillarp (1947), Rogers and Williams (1947) (and see review by Anderson, 1948), supported Gomori's opinions. Boscott and Mandl (1949) used the amine-aldehyde condensation of Oster and Mulinos (see below) to block the reaction of the adrenal cortex with 2 : 4-dinitrophenylhydrazine. They showed, *in vitro*, that the reagents used (sulphanilic acid or sulphonamide) would not react with either dehydro*iso*androsterone or with deoxycorticosterone acetate. If either of these, or similar steroids, remained in the sections they should have given a positive phenylhydrazine reaction according to these authors. The extraction experiments of Olson *et al.* (1944) showed that the ketosteroid content of the tissues is minute, and Rogers and Williams (1947) calculated that 0·017 μg. of ketosteroid might be present in an average frozen section of adrenal. They considered that such an amount could not possibly be demonstrated by the phenylhydrazine reaction even if condensation took place. This view overlooks the fact that the local concentration of the steroid hormones and their inactive precursors may be very high. If all the steroid in the adrenal cortex was present in a single layer of cells its concentration per cell might be high enough to give a positive histochemical reaction. These arguments were considered at length by Deane and Seligman (1953) in their review of the known procedures for the cytological localization of ketosteroids.

Naphthoic Acid Hydrazide

The naphthoic acid hydrazide-dianisidine (NAHD) reaction was originally described as specific for ketosteroids and was employed for this purpose particularly upon frozen sections of adrenal glands. Camber used watery solutions, both for the hydrazide and for coupling, and inserted a short period of oxidation with iodine between cutting the sections and immersion in the hydrazide solution. He noted that this manœuvre improved the final colour considerably. In the light of our knowledge of the plasmal and pseudoplasmal reactions this point is of particular significance, suggesting the production of lipid aldehyde groups by oxidation with iodine. Ashbel and Seligman used watery solutions in their earlier experiments but rejected these later as giving inferior results. They subsequently employed 50 per cent. alcoholic solutions, buffered at pH 7·5 for the final coupling with diazotized dianisidine. Fig. 98, p. 337, shows the results of the NAHD reaction in human adrenal cortex.

These authors were well aware of the fact that naphthoic acid hydrazide would condense with aldehydes and they realized that the specificity of the NAHD reaction for ketosteroids depended on whether tissue aldehydes were involved or not. They maintained, however, that this point depended on whether the hydrolysis of plasmalogens to plasmal occurred under the conditions involved although, in fact, the chief aldehydes present would be those formed from lipids during formalin fixation.

In answering the question to their own satisfaction, Ashbel and Seligman made much of the statement by Hayes (1949) that exposure of tissues to formalin for more than 6 hours would hydrolyse plasmalogen to plasmal, but that further exposure would destroy all the liberated plasmal. In the case of ketosteroids, these authors claimed that the action of formalin was absolutely necessary for unmasking the reactive carbonyl group, but once this had been accomplished (1 or more hours) deterioration of the staining reaction did not occur with further exposure to formalin (weeks). Feldman (1950), however, found that 48 hours' treatment with formalin was necessary to produce the reaction in sections of adrenal cortex, and that after 3 months' exposure the cortical cells ceased to react either with leucofuchsin or with NAHD. My own results confirm the gradual deterioration of the NAHD reaction with prolonged formalin treatment, but I find that 18 hours in formalin, and probably an even shorter time, is quite sufficient to produce a strong reaction. The explanation offered by Ashbel and Seligman in their denial of the participation of plasmal in the NAHD reaction overlooked the important fact, established by Cain (1949a), that oxidation of the double bonds in unsaturated fatty acids by atmospheric oxygen produces what this author has termed the pseudo-

plasmal reaction. Autoxidation of fatty acids to aldehydes proceeds quite readily in solutions of formalin and there is every likelihood that such aldehydes are responsible for the positive Schiff and NAHD reactions in sections of adrenal fixed even for short periods in formalin.

Three further pieces of evidence in favour of the specificity for ketosteroids of the NAHD reaction were offered by Ashbel and Seligman, however. The first was the fact that the four monoketosteroids, which they used for *in vitro* tests, failed to recolorize Schiff's solution, though three of them (testosterone, œstrone and pregnenolone) representing 3, 17 and 20 ketosteroids, combined readily with NAH. This was interpreted to mean that while Schiff's reagent revealed aldehydes only, NAHD revealed both aldehydes and ketones. Ashbel and Seligman noted in their experiments that no reaction with Schiff's reagent occurred in control sections of adrenals fixed for a longer or shorter period in formalin. This observation is quite inexplicable ; Feldman's results and my own agree in showing that a positive Schiff reaction invariably occurs in control sections. I do not, however, agree entirely with Feldman's statement that the NAHD and Schiff reactions give identical results, and the lack of identity which I observed was more striking when certain other tissues, such as brain, were examined.

The second piece of evidence (Seligman and Ashbel, 1951) was the observation that excess sodium *o*-sulphobenzaldehyde could cause reversal of the reaction of NAH with both ketones and aldehydes *in vitro*. But whereas in the case of ketones reversal occurred rapidly, in the case of aldehydes it was relatively slow. This reaction was applied to tissue sections, and nervous tissue which had been treated with the hydrazide was incubated for 24 hours at 22° in a solution of 5 per cent. sulphobenzaldehyde. After washing in dilute sodium bicarbonate and in water for 24 hours, subsequent treatment with the diazonium salt gave an intensity of staining " strikingly less " than that observed in the controls. The authors suggested that this indicated that the carbonyl group in formalin-fixed nervous tissue was ketonic in nature. The specificity of this reversal reaction is open to doubt, since the intensity of staining is appreciably reduced by a similar process in the absence of sulphobenzaldehyde. The third piece of evidence produced by Seligman and Ashbel was the negative reaction obtained by the phenylhydrazine-formazan method. As already stated, a positive reaction certainly indicates aldehydes and excludes ketones but a negative reaction has no significance. The specificity of the NAHD reaction for ketones still depends on whether aldehydes are or are not present in the frozen sections employed. Where they are known to be present ketones cannot be distinguished, even in amounts which are capable of giving a positive NAHD reaction.

Salicyloyl Hydrazide

Camber (1954, 1957) introduced salicyloyl hydrazide as a fluorescent reagent for aldehydes and ketones. He observed that the hydrazones formed by this compound with aldehydes gave brilliant fluorescence in many colours when exposed to U-V light. Its ketonic hydrazones gave a uniform dull blue fluorescence. The successful use of this reagent in histochemistry has not yet been reported. A non-fluorescent hydrazide (β-resorcylic acid hydrazide) was prepared by Camber and Dziewiatskowski (1951) for use as an alternative to naphthoic acid hydrazide. Its hydrazones can be coupled with diazonium salts to give purple or red compounds. These might be used for the histochemical localization of tissue carbonyls but it is doubtful whether specificity for ketones can be achieved.

Histochemical Demonstration of Ketosteroids by a Battery of Reactions

Although it is certain that no single reaction is sufficiently specific for ketosteroids to be used for the histochemical demonstration of these in tissue sections, neverthcless certain investigators (Deane and Greep, 1946 ; Dempsey and Wislocki, 1946 ; Dempsey, 1948 ; Greep and Deane, 1949) maintained that the ketosteroid content of the adrenals could be made visible by means of a battery of reactions and, furthermore, that a quantitative appreciation could be made. The seven reactions or properties concerned were as follows :

Sudanophilia
Acetone solubility
Birefringence
Fluorescence
Liebermann-Burchardt reaction
Schiff reaction (plasmal or pseudoplasmal)
Di-nitrophenylhydrazine reaction.

To these Greep and Deane added the ammoniacal silver reaction. In addition to criticisms of the specificity of thc individual methods it may be noted, in opposition to these views, that in the adrenal cortex the above reactions do not occur in a single site. The larger, acetone-soluble, fat droplets are sudanophil, occasionally birefringent and Liebermann-Burchardt positive, but they are Schiff and di-nitrophenylhydrazine negative. The smaller Schiff, phenylhydrazine and, incidentally, NAHD-positive granules may give all the above reactions except that they are uniformly isotropic. Yoffey and Baxter (1947) stated that birefringence was not a property of the fat droplets in fresh adrenal cortex. With the passage of timc, however, birefringent crystals appeared, and these were abundant in formalin-fixed sections.

By means of X-ray studies they were unable to identify the crystals either as cholesterol or as ketosteroids. The evidence in Feldman's paper suggested that a positive reaction to the entire battery of tests was never present in a single locus, and Boscott and Mandl (1949), to my mind correctly, maintained that an artificial mixture of tissue constituents, such as neutral fats, cholesterol, carotenoids, tissue aldehydes and phospholipids, would satisfy all the tests even if no ketosteroids were present. Gomori (1952), in a paper on the histochemistry of lipid carbonyl groups, expressed substantially the same views.

Nevertheless Deane and Andrews (1953), observing that in formalin-fixed frozen sections the stainable droplets contained a mixture of pseudoplasmals and ketones, drew attention to the remarkable parallelism between the two in steroid producing organs. Deane and Seligman (1953) maintained that the carbonyl reactivity of the droplets in the adrenal cortex could be related to physiological activity of the gland. These concepts were examined by Karnowsky and Deane (1955) who studied the development of aldehydes in lipid droplets by chemical and histochemical means. Observing that the autoxidation of lipids was enhanced by metals these authors added a number of chelating agents to their fixative (buffered formalin) and found that most of these reduced the intensity of the Schiff reaction to 50–75 per cent. of that shown by control sections. Dithizone and 8-hydroxyquinoline were more effective than versene and sodium citrate in this respect. A more marked reduction in the Schiff reaction was obtained by adding antioxidants to the fixative. Hydroquinone, pyrogallol and nordihydroguaretic acid were all effective.

In no case did Karnovsky and Deane find the NAHD reaction positive when the Schiff reaction was negative. When the latter was weak the former was often negative, indicating a lower degree of sensitivity. They concluded that histochemical methods do not detect ketosteroids in the droplets of the adrenal cortex and that, except for a small amount of plasmalogen, all the detectable lipid carbonyl groups of the adrenal cortex are artifacts caused by autoxidation of unsaturated fats. All this does not mean that the reactions cited cannot be used for morphological correlation of changes in the adrenal cortex in various conditions. I consider, however, that at the present time a better indication of the state of the adrenal cortex is given by the application of coupling azo dye techniques, particularly for alkaline and acid phosphatase, and other techniques for β-glucuronidase (see Chapters XIV, XV and XVI), although the use of these methods is at present just as empirical as is the use of carbonyl methods.

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CHAPTER XIII

THE PRINCIPLES OF HYDROLYTIC ENZYME HISTOCHEMISTRY

General Survey of Enzyme Histochemistry

In the first (1953) edition of this book the following statement appeared in the preface to the section of four chapters on enzyme methods. "Although a few years ago only 2 or 3 enzymes could be demonstrated in the tissues by histochemical means, there are now techniques for at least 18." In the last five years advances in the field of enzyme histochemistry have been so great that the sentence quoted must now be amended to read "for at least 45." This rapid expansion has been accompanied by improvements in technique affecting the majority of the enzymes recorded in the 1953 list. At that time it was notable that the majority of methods concerned the hydrolytic enzymes, mainly phosphatases and carboxylic acid esterases, and that other enzyme groups were poorly represented. No methods existed for the various amino-acid oxidases, urease, arginase or peptidases. Now, five years later, methods have been described for D-amino-acid oxidase and leucine aminopeptidase, while enzymes of the cathepsin class can be demonstrated by a combination of existing methods with suitable activators and inhibitors.

Considered against a grand total of some 700 recorded enzymes (Dixon and Webb, 1958) the histochemical total of less than 50 may not seem impressive. It is necessary, however, to consider the matter in relation to general coverage of enzyme systems rather than on a purely numerical basis. Viewed in this light we see that in the hydrolytic enzyme group the *esterases* are very well covered, the *phosphatases* and *peptidases* less so, and the *carbohydrases* (glucosidases) better than might be expected. The deaminases and deamidases (glutaminase, asparaginase and urease) are still not represented except for *phosphamidase* in the second of these two classes. In the group of phosphorylases only a so-called amylophosphorylase appears in the list, but there is recent evidence (Takeuchi, 1958) that a branched-chain as well as a straight-chain synthesizing enzyme can be demonstrated by the current histochemical method. In the group of adding enzymes (e.g. *catalase*, *carbonic anhydrase*, *aldolase*, fumarase, aconitase and the amino-acid decarboxylases) there are histochemical methods for the first three only. All these methods, however, are either technically suspect or of dubious specificity.

In the large group of transferring enzymes the division of oxidizing and reducing enzymes now contains many histochemically demonstrable members. The *peroxidases* are represented by the enzyme which gives its name to the whole sub-division and the aerobic oxidases, by *cytochrome oxidase* and *tyrosinase*. The aerobic dehydrogenases are somewhat inadequately represented by D-*amino-acid oxidase*, but other enzymes in this division will doubtless be added in the near future. In the division of flavoprotein enzymes (reduced co-enzyme dehydrogenases) the so-called *DPN and TPN-diaphorases* can be demonstrated and the anærobic *dehydrogenases* are probably better represented histochemically than any other class of enzyme.

We see, therefore, that histochemical coverage of the various enzyme classes is now very much better than it was only a few years ago. There is, of course, no room for complacency, but it is possible to feel that in the near future enzyme histochemistry will come to occupy an important place in relation to enzymology and enzyme biochemistry, and that its application to pathology in particular will extend the horizons of that science far beyond the level at which they have remained fixed since Virchow published his great work, *Cellular Pathology*, 100 years ago.

A list of the available techniques in enzyme histochemistry is given in Table 25, below, and against each enzyme appears the number of different methods applicable to its localization. These are in all cases individual methods, not modifications of a single one, and their inclusion in the Table is not necessarily an indication that they are satisfactory in practice, or that the localization achieved is accurate.

TABLE 25

Alkaline phosphatases	3	Amine oxidase	2
Acid phosphatases	3	Choline oxidase	1
Desoxyribonuclease	1	D-Amino-acid oxidase	1
5-Nucleotidase	1	DPNH-diaphorase	1
Adenosine triphosphatase	1	TPNH-diaphorase	1
Aldolase	1	Succinate dehydrogenase	1
Glucose-6-phosphatase	1	Glucose-6-phosphate dehydrogenase	1
Phosphorylase	1	6-Phosphogluconate dehydrogenase	1
Amylo-1,4-1,6-transglycosidase	1	β-Hydroxybutyrate dehydrogenase	1
Pyrophosphatase	1	Alcohol dehydrogenase	1
Phosphamidase	2	Lactate dehydrogenase	1
Lipases	1	Isocitrate dehydrogenase (TPN)	1
Carboxylic acid esterases	4	Isocitrate dehydrogenase (DPN)	1
" AS-type " esterases	1	Glutamate dehydrogenase	1
Acetylcholinesterases	2	α-Glycerophosphate dehydrogenase	1
Pseudocholinesterases	2	Malate dehydrogenase	1
Phenol sulphatase	3	α-Hydroxysteroid dehydrogenase	1
β-Glucuronidase	3	β-Hydroxysteroid dehydrogenase	1
β-Galactosidase	2	Cytochrome oxidase	1
β-Glucosidase	1	Tyrosinase	1
Acetyl-β-glucosaminidase	1	Leucine aminopeptidase	1
Carbonic anhydrase	1	Cathepsins	1
Peroxidase	2	Catalase	1

Of the enzymes listed above those which concern phosphate esters, and which operate at neutral, alkaline or acid pH levels, will be considered in Chapters XIV and XV. The carboxylic acid esterases are dealt with in Chapter XVI, and the glycosidases in Chapter XVII. The various oxidases, irrespective of type, are described in Chapter XVIII and the dehydrogenases and diaphorases in Chapter XX. The principles of dehydrogenase histochemistry, which differ in many important respects from those of the hydrolytic enzymes, are considered separately in Chapter XIX, rather than in this chapter. A miscellaneous group of enzymes, comprising the peptidases, the sulphatases, and carbonic anhydrase, appears in Chapter XXI.

The main subject-matter of this chapter concerns the general principles of enzyme histochemistry as they appear at the present time. Since this book first appeared efforts have been made to develop various branches of enzyme histochemistry in a manner less empirical than was customary in the past and, taken together, these efforts permit a set of principles to be drawn up which can stand as a guide for future work. It is probable that for some time to come the empirical approach to the development of new methods will continue to be used in enzyme histochemistry. As soon as possible, however, all methods should be subjected to critical evaluation, and it is to be hoped that studies of the type discussed in this chapter will ultimately be universally applied.

General Principles of Hydrolytic Enzyme Histochemistry

Although many individual authors have contributed to the development and understanding of the various techniques of enzyme histochemistry the most significant contributions, from the theoretical point of view, have come from Holt (1956, 1958) and his collaborators, and from Seligman and his associates in Boston and Baltimore. Some of my own work (Pearse, 1954 ; Defendi and Pearse, 1955) has also been concerned with the theoretical basis of enzyme histochemistry. The whole matter was admirably set forth by Nachlas, Young and Seligman (1957) in their contribution to the symposium on *Localization in Histochemistry* held at the Eighth Annual Meeting of the Histochemical Society in Baltimore, and I have used their text as a basis for much of the discussion which follows.

Preparation of Tissues

Although the question of fixation and the preservation of enzymes has already been considered in Chapter IV, certain additional problems remain. The first of these is the question of selective loss of enzyme

during processing, whether this involves fixation or not. That such selective loss in fact occurs was shown as long ago as 1950, by Mængwyn-Davies and Friedenwald, in the case of alkaline phosphatases. It may affect part of a single enzyme, less strongly bound than the part remaining in the tissues, or more selectively a single specific enzyme within a group having wider specificity for the histochemical substrate. No hard and fast rules can be laid down for the processing of tissues for different histochemical enzyme techniques but, broadly speaking, two things are of paramount importance. These are the preservation of the maximum possible amount of enzyme activity and the maintenance of the *in vivo* localization of the enzyme. Adherence to these two principles results essentially in some form of compromise since, to take the case of a soluble enzyme, preservation of localization by fixation must result in a reduction of enzyme activity. In dealing with soluble enzymes, fixation of some sort is absolutely necessary, although it can sometimes take the form of simultaneous fixation, with the fixative included in the incubating medium. Up to 3 per cent. of formalin can be included in this way or, under other circumstances, up to 50 per cent. ethanol or acetone. Holt (see Holt and O'Sullivan, 1958) has frequently drawn attention to the paradox that perfect preservation of morphology and enzyme activity may lead to less accurate localization of enzyme by a given cytochemical method. This must be understood and accepted, but it must also be appreciated that the converse is not necessarily true, and that the loss of accuracy may in any case be only an apparent one. Histochemists trained as histologists must always avoid the too facile description of a sharper and clearer microscopical image as *better* enzyme localization. These points will be elaborated at the end of this chapter in considering the criteria by which the histochemist may assess the accuracy of his results.

If fixation can be avoided altogether, as it can when the enzyme system concerned is localized within certain intracellular organelles, new principles for handling of the tissues can be formulated. These are included in the comprehensive term " protection " which describes procedures developed initially by Scarpelli and Pearse (1958) for the preservation of Krebs cycle and other enzymes in mitochondria, and later extended to cover non-mitochondrial systems as well. Novikoff (1957) first extended the use of substances employed for preserving mitochondria in homogenates to their preservation in tissue sections, and protection of tissues *after* cold formalin fixation has been used by Holt and Withers (1958). These authors fixed their tissue blocks in cold formol-calcium solutions and then transferred them to ice-cold 0·88M-sucrose containing 9 g./litre of gum acacia. The blocks were allowed to remain in this solution at 0°–2° for 24 hours and then cut

on the freezing microtome. Table 26, below, sets forth the various types of protection, any or all of which it may be necessary to apply in order to ensure preservation of a given enzyme system in tissue sections. Certain criteria here set forth are not valid for the preservation of enzymes in free floating cells or in smears.

TABLE 26

Type of Injury	Cause	Effect	Remedy
ANOXIC .	Deprivation of blood supply.	Mitochondrial damage.	Minimum delay in handling fresh tissues.
THERMAL	Storage at ambient temperatures.	Reduced enzymo activity.	Maintain sections at 0–4° until required. Blocks at −20° or better at −70°.
	Freezing and thawing.	Loss of enzyme, co-factors, etc.	None, except use of smears.
	Warming of sections drying on storage.	Loss of onzymo.	Keep sections in moist air at 0–4°.
OSMOTIC .	Swelling of mitochondria, etc.	Loss of soluble enzymes. Destruction of mitochondria.	Protection by 0·88M-sucrose, or dextrin, or 7·5 per cent. PVP.
	Increased permeability.		
CHEMICAL	Loss of activators, co-factors, etc.	Reduced enzymo activity.	Replacement of lost factors. Alternative factors. Mg^{2+}, ATP, Mn^{2+}, Co^{2+}.

Preservation of Enzymes in Fresh Tissues

The first type of injury occurs when mitochondria are deprived of their supply of oxygen. Sensitive mitochondria show signs of increased permeability of their membranes almost at once. Insensitive mitochondria, such as those of cardiac and voluntary muscle, may show no changes for a considerable period of time. In practice this means that when intra-mitochondrial enzyme systems are being studied, *post mortem* tissues are unsuitable for anything except relatively crude studies at histological levels. Even material from the operating theatre may show severe damage to sensitive mitochondria if the interval between tying off the blood vessels and removal of the material is more than a few minutes. Needle or drill biopsy tissues are excellent.

The second type of injury, described in the table as thermal, occurs first if the tissues are left at room temperature. This can be avoided by chilling the tissues or by quenching small pieces at once at −78° by the application of dry ice or by immersion of a metal tissue holder in a dry ice-acetone mixture. Unfortunately this manœuvre also causes thermal injury to mitochondrial enzyme systems. Damage due to freezing at −78° was assessed by Porter *et al.* (1953) in the case of a number of enzyme systems. They found, for instance, that less than

one-fifth of the succinoxidase activity of a tissue homogenate survived rapid freezing and subsequent thawing and maintained that slow freezing to −10° caused less damage to enzymes, although ice crystal growth was much greater. The effects noted by Porter and her associates cannot be reproduced when histochemical tests for soluble and insoluble mitochondrial enzyme systems are applied to sections from rapidly quenched (−78°) and slowly frozen (−12°) tissue blocks. Using the DPN-linked L-Malate dehydrogenase system for instance, with the substrate concentration at 10^{-2} M and DPN at 10^{-2}, 10^{-3}, and 10^{-4} M, enzyme activity decreases in the same order of decreasing DPN concentrations after quick or slow freezing. Moreover, the overall activity is less in slowly frozen material and the enzyme is localized entirely outside the mitochondria, indicating a severe degree of damage. Tissue sections cut on the cold microtome are adequately preserved for short periods at 0–4° or at −20° in moist atmospheres. Blocks of tissue can be stored at −20° for longer periods, perhaps up to 7 days or so, but for adequate long-term preservation temperatures as low as −70° are necessary. The design and production of an inexpensive low-temperature storage cabinet is long overdue.

Part of the damage described as due to freezing may well be due to the subsequent thawing which precedes incubation. With fresh frozen sections additional damage may be caused by the passage of the knife through the tissues, since this is preceded by a melting zone and followed by refreezing (see Chapter II, p. 21). It is probable, therefore, that double freezing and thawing is inevitable in the case of fresh frozen sections.

In addition to the damage caused by thawing after freezing further damage to intramitochondrial enzymes is produced by drying the sections (mounted on slides or coverslips) and allowing them to remain at room temperature before proceeding to incubation. This can be avoided by storage in a cold (0 to −20°) moist environment.

The third type of damage, to which mitochondria and other intracytoplasmic organelles are particularly sensitive, is described in the table as osmotic. This occurs in several stages, of which the final and most severe stage involves disruption of the mitochondria. It can be avoided by the use of hypertonic media. Of these, polyvinyl pyrrolidone (PVP) with or without additional sucrose, as used by Novikoff (1956), seems to be the most suitable for general use.

The final type of injury, described in Table 26 as chemical, comprises a miscellaneous group of circumstances. During processing many smaller molecules may become dissociated from their situation, and during subsequent incubation they are lost. Metallic ions functioning as activators may come into this category and their replacement is necessary for restitution of enzyme activity. Other factors, such as

ATP and Mg^{2+}, may have to be added in order to assist in the preservation of mitochondrial structure, although there is no evidence that these have been lost in processing.

The protection of cytoplasmic structures, in which the enzyme systems are localized, from damage during processing and during incubation must be given far more consideration in modern enzyme histochemistry than it has received in the past. In many cases, the application of the principles of protection to histochemical practice results in enzyme localizations which agree closely with the best results obtainable by the techniques of other disciplines.

Histochemical Reactions for Hydrolytic Enzymes

The four types of histochemical reactions for hydrolytic enzymes are :

(1) Simultaneous capture (coupling, conversion, chelation).
(2) Post-incubation coupling (chelation, conversion).
(3) Self-coloured substrate (solubility change).
(4) Intramolecular rearrangement.

Of these four types the first is of outstanding importance, the second of somewhat lesser importance, and the third and fourth are confined, at present, to a few isolated examples. The simultaneous capture principle operates in the older metal salt precipitation techniques, such as those for alkaline and acid phosphatases, and in the azo dye methods for various hydrolytic enzymes (Chapters XIV, XV, and XVI) when the coupler may be either naphthol or naphthylamine. It operates also in the oxidation of liberated indoxyls to indigos (Chapter XVI) and in the metal chelation of formazans from mono and di-tetrazolium salts (Chapter XX), although in this case the kinetics are not those of the more usual capture reactions.

Simultaneous Capture

In discussing reactions based on the simultaneous capture principle it is expedient to consider first the coupling azo dye methods, since in the evolution of these have arisen most of the concepts on which modern enzyme histochemistry is based. With these methods, in which a diazonium salt present in the incubating medium combines with the coupler as soon as the latter is released from the substrate, the main obstruction to accurate localization of the responsible enzyme is diffusion of the primary reaction product (PRP). This is likely to be governed by several factors; by the rate of hydrolysis of the substrate, by the diffusion coefficient of the PRP for the buffer system and pH employed, and by the rate of coupling of PRP and diazonium salt

(Defendi and Pearse, 1955). All three can be controlled to some extent ; first, by modifying the substrate, which affects the rate of hydrolysis, the diffusion coefficient and the coupling rate and, secondly, by modifying the diazonium salt. This may affect the rate of hydrolysis (by inhibition) and the rate of coupling. If the PRP has an affinity for protein this quality of "substantivity" will obviously affect its rate of diffusion. Substantivity of the PRP is a quality which is certainly desirable in designing post-incubation coupling methods (see below) and, according to many authorities, it is desirable also in simultaneous capture reactions. I do not altogether agree with this latter view myself, preferring whenever possible to obtain rapid capture of a soluble non-substantive PRP. This preference is based on practical rather than on theoretical experience.

In order to assess the importance of the coupling rate in simultaneous capture azo dye reactions experiments were made (Pearse, 1954 ; Defendi and Pearse, 1955) to measure the rate under various conditions. Using a continuous flow apparatus and photometric control, we tested the coupling rates of α and β-naphthol, and of a number of substituted naphthols, with tetrazotized di*ortho*anisidine (Fast blue B salt) at various pH levels. The half time (t) for coupling with α-naphthol varied from 92 millisecs at pH 9·2 to 750 millisecs at pH 5·3. For β-naphthol the figures were 80 millisecs at pH 9·2 and 3 minutes 15 seconds at pH 5·8. As published, the velocity constants for the reactions were not strictly accurate since k was calculated from the usual formula for a bimolecular reaction.

$$k = \frac{1}{t(a-b)} \log_n \frac{b(a-x)}{a(b-x)}$$

where a and b are initial concentrations of the reactants and x the amount of the product formed.

This would be in order for the coupling of naphthols with monodiazonium salts, but not with bisdiazonium salts like Fast blue B, where the initial stage of the reaction may be supposed to be monocoupling followed by a second coupling at the other end of the molecule. The error entailed, however, is not very great and, since our results showed a linear relationship between pH and log k, their significance remains as stated in the original papers. For both naphthols there were critical pH levels below which the coupling rates fell off rapidly (Fig. 99). The greater efficiency of α-naphthol on the acid side of neutrality was reflected in the histochemical results which were carried out in parallel. It was suggested that the coupling rate was of importance in histochemical azo dye methods in the case of the more soluble naphthols. but of less importance with the relatively insoluble ones in producing accurate enzyme localization.

More recently (Nachlas *et al.*, 1959) have used a similar apparatus to measure the coupling rates of a series of naphthols and naphthylamines with a number of different diazonium salts, in order to test the effect of various substituent groups in both diazonium salt and coupler. Plotting log k ($\frac{1}{2}$ time) against σ, the substituent constant for the

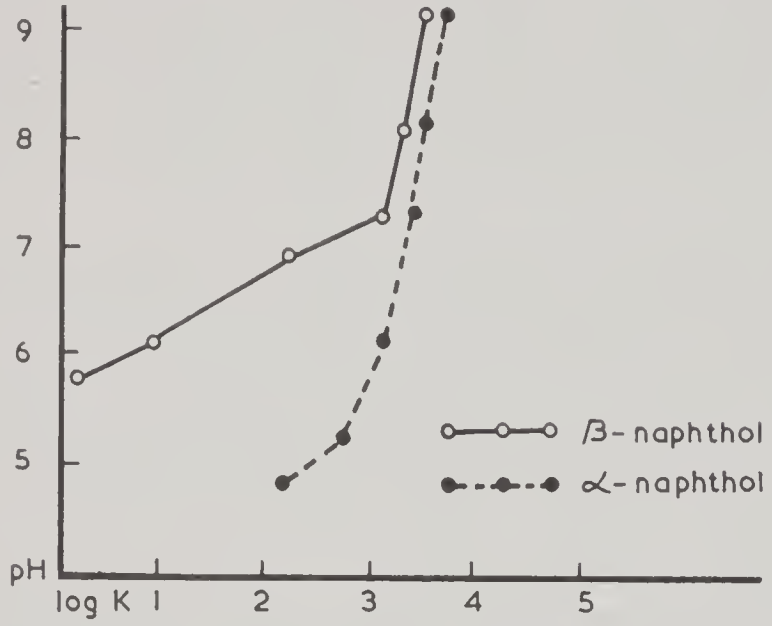

Fig. 99. Coupling of Fast Blue B with Naphthols as a function of pH.

particular functional group (Hammett, 1953), they found that as substituents were added to increase coupling efficiency the rate of coupling for the naphthols continued to increase while that for the naphthylamines levelled off sharply. Fig. 100, below, illustrates this effect.

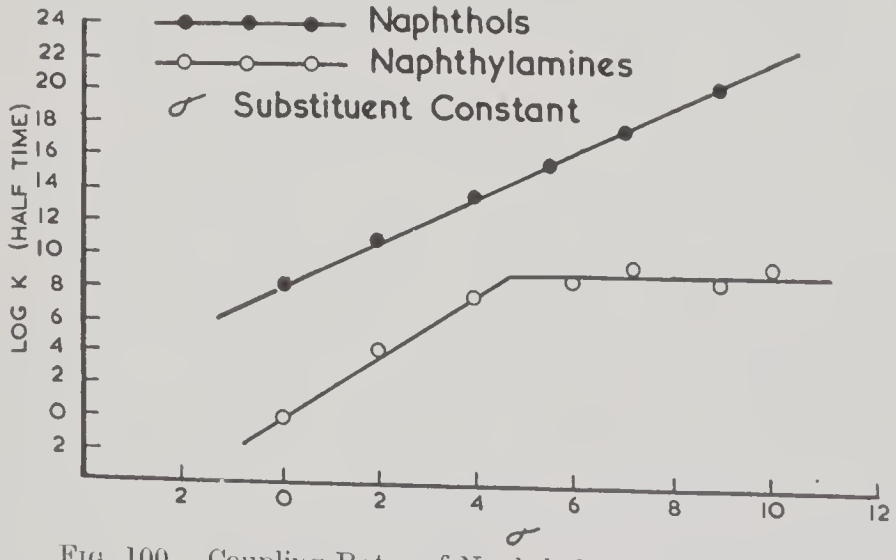

Fig. 100. Coupling Rates of Naphthols and Naphthylamines.

This important observation explains the histochemical differences in performance of naphthol and naphthylamine primary reaction products and forms a basis for new efforts to improve existing methods in the coupling azo dye field of enzyme histochemistry.

Kinetics of Simultaneous Capture Reactions

The kinetics of enzyme histochemistry, particularly as applied to the indoxyl acetate methods (Chapter XVI, p. 467), have been considered by Holt and his co-authors (Holt, 1956, 1958 ; Holt and O'Sullivan, 1958) in a comprehensive series of papers. Their findings and conclusions, which are reported extensively below, are applicable directly to simultaneous capture reactions of many different types. They have less direct application to the simultaneous chelation methods (Pearse, 1957) and may require extensive modification in respect of enzymes enclosed in such box-like structures as mitochondria or lysosomes.

For the purpose of theoretical treatment considerations of histochemical enzyme reactions were divided by Holt into those of the initial process, followed by considerations of the rate of production of the primary reaction product (PRP) the formation of the final reaction product (FRP) and the life of the PRP, and lastly, those of variations in distribution of the FRP. This order is the order in which they are here presented.

The Initial Process. It is necessary, in the first instance, that both the substrate and the capture reagent should penetrate rapidly into the tissues, and for this purpose it is desirable that both should be sufficiently soluble in water to provide high concentrations in the incubating medium. For rapid diffusion of substrate and capture reagent both should be of *low molecular weight* and also of *low polarity*, so that they can pass through lipid layers and avoid adsorption by protein. Holt and O'Sullivan (1958) made theoretical studies of simplified models in which the tissue was regarded as a homogeneous entity containing an enzyme site. Permeability barriers were ignored and depletion of substrate considered as negligible. Calculations of the time taken for the concentration of a substance at the centre of a tissue slice to reach 90 per cent. of the external value showed that for good penetration thin tissue slices were essential. High external concentrations of substrate and capture reagent were also shown to be essential, with high diffusion constants in both cases. It was suggested that the diffusion constant D′ should preferably be greater than $10^{-6}cm^2s^{-1}$ and the molecular weight of the substrate, therefore, below 1,000. In this context it is to be noted (Neale, 1936) that the diffusion constants of many dyestuffs in polysaccharides and proteins are up to 10^4 times smaller than in aqueous solution. In Table 27, below, are given the times (in seconds) for the concentration of a substance at the centre of the tissue slice to reach 90 per cent. of the external value.

Holt and O'Sullivan commented that the desirable non-bonding substances are usually non-polar and therefore lipid soluble. This

TABLE 27

(After Holt and O'Sullivan)

D' (cm^2s^{-1})	Section Thickness						
	1μ	5μ	10μ	15μ	20μ	50μ	500μ
10^{-5}	0·00025	0·00625	0·025	0·056	0·1	0·625	62·5
10^{-6}	0·0025	0·0625	0·25	0·56	1·0	6·25	625
10^{-7}	0·025	0·625	2·5	5·6	10·0	62·5	6,250
10^{-8}	0·25	6·25	25·0	56·0	100·0	625	62,500

enables them to penetrate intact lipid layers in the cells and tissues. Collection of the substrate in specific sites, as by preferential adsorption, is manifestly undesirable and it is obvious that in biological systems many other (uncontrollable) factors will modify the assumptions made above.

Rate of Production of the Primary Product. When the concentration of a substrate is high in relation to that of enzyme it is removed by a reaction which obeys zero order kinetics. That is to say, the rate of the reaction is independent of the substrate concentration and related solely to enzyme concentration. If, however, the substrate is present in low concentration the kinetics may become first order. That is to say, they may be related directly to the concentration of substrate. Holt suggested that it was reasonable to suppose that rapid ingress of substrate into tissue sections gives rise to zero order production of the PRP. This being the case he considered the situation at a highly active site, into which the substrate could diffuse rapidly, and concluded that zero order kinetics were in fact valid for a wide range of conditions *irrespective of the activity of the site.*

Formation of the Final Reaction Product and Life of the Primary Reaction Product

Continuing the foregoing investigations Holt and O'Sullivan studied the simultaneously occurring processes of PRP formation, PRP diffusion and removal of PRP by conversion to FRP. Diffusion and capture are the two methods by which the PRP is removed from the reaction and, for the purposes of calculation, it was assumed that the former obeyed Fick's law. Since the concentration of free PRP is likely to be low at any given point, if the concentration of the capture reagent is high the reaction between them should obey first order kinetics and be dependent solely on capture reagent concentration. This proposition assumes that the capture reaction is irreversible and that the FRP is the sole product. According to Holt and O'Sullivan the presence of several stages in the capture reaction introduces no complications provided the overall kinetics are first order. If the

capture reagent is not present everywhere in adequate excess, however, the reaction will deviate towards second order kinetics. In this case quantitative theoretical study is not possible.

From the above considerations it may be deduced that the smaller the diffusion constant of the PRP, and the larger the velocity constant of the capture reaction, the more precise will be the localization of the FRP.

In earlier papers O'Sullivan (1955, 1956) described a model system in which the enzyme was presumed to be localized in a spherical site of known radius set in an infinite medium. It was assumed that the substrate diffused rapidly and that the FRP was completely non-diffusible. A measure of the degree of localization achieved was derived theoretically and described as the *localization factor*. This is now defined as the ratio of the mass of FRP actually deposited in the site in a given time to the mass of FRP that would be produced in the given time, if the PRP were converted *quantitatively* into FRP in that time. The localization factor thus defined is dependent on the radius of the site, the velocity constant of the capture reaction and on the diffusion coefficient of the PRP. Provided experimental values for these three can be obtained the localization factor provides a means of comparison between different cytochemical methods. For convenience Holt and O'Sullivan defined the localization factor as the percentage of total FRP which is to be found in the site of enzyme activity and their results are given in the two tables which are reproduced below. The first of these shows the relationship of the localization factor to the diffusion constant of the PRP and to the velocity constant of the capture reaction for a site of radius 1μ. This the authors took as the practical limit of resolution of the light microscope in this context.

TABLE 28

Variation in Percentage of FRP in a Site of Radius 1μ

Diff. Constant ($cm^2\ s^{-1}$)	Velocity Constant of Capture Reaction (s^{-1})							
	10^5	10^4	10^3	10^2	10	1	10^{-1}	10^{-2}
10^{-5}	85·1	57·2	19·0	3·5	1·4	10^{-1}	10^{-2}	10^{-3}
10^{-6}	95·3	85·1	57·2	19·0	3·5	1·4	10^{-1}	10^{-2}
10^{-7}	98·5	95·3	85·1	57·2	19·0	3·5	1·4	10^{-1}
10^{-8}	99·5	98·5	95·3	85·1	57·2	19·0	3·5	1·4
10^{-9}	99·9	99·5	98·5	95·3	85·1	57·2	19·0	3·5
10^{-10}	100	99·9	99·5	98·5	95·3	85·1	57·2	19

The second table (Table 29) gives the calculated values for the localization factor for sites of different size, for various combinations of velocity constant and diffusion constant.

TABLE 29

Variation in Percentage of FRP with Site Radius

Site Radius (μ)	Ratio Velocity Constant/Diffusion Constant (cm^{-2})					
	10^{12}	10^{11}	10^{10}	10^{9}	10^{8}	10^{7}
0·1	85·1	57·2	19·0	3·5	1·4	10^{-1}
1·0	98·5	95·3	85·1	57·2	19·0	3·5
10·0	99·9	99·5	98·5	95·3	85·1	57·2

Holt and O'Sullivan considered that for methods depending on stain density at least 50 per cent. of the FRP should be deposited in the site of enzyme activity, and Table 29 shows that with a site of radius 10μ a velocity constant for the capture reaction of 10 s^{-1} is sufficient. With sites of radius less than 1μ, however, velocity constants of 10^3 s^{-1} and greater are necessary. According to Cotson and Holt (1958) the velocity constants of cytochemical enzyme reactions are never higher than 10 s^{-1} and the authors deduced from this that the minimum radius of sites in which *quantitative* enzyme localization could be achieved is about 10μ, when the PRP is freely diffusible. They therefore maintained that the diffusion constant of the PRP must be reduced and that this could best be brought about by arranging for the PRP to possess substantivity for protein. This concept is further discussed below in the section dealing with the properties of the PRP.

Variation of Stain Distribution with the Localization Factor

Localization factors discussed above give a measure of the relative amounts of FRP within and without sites of different radii under different conditions. They do not indicate the actual distribution pattern of the FRP. This has to be considered if a full interpretation is to be made of the significance of localization factors in relation to the microscopic examination of tissues after the performance of histochemical enzyme reactions.

Holt and O'Sullivan (1958) therefore described a further factor the *specific stain density*. This can be defined as a density of FRP such that, if perfect localization is achieved, its value is unity throughout the enzyme site and zero elsewhere. The specific stain density (s) varies with the value of the localization factor (F) and with a factor ρ which is the ratio of the radius of an arbitrary spherical zone around the site to the radius of the site itself. These relationships are shown in Fig. 101.

It is evident from the above graph that if precise localization of an enzyme is desired the value of F should certainly be higher than 0·65.

Holt considered that a reasonable picture of the site might still be obtained with much smaller F values, but that values below 0·01 should not be tolerated with any method. His calculations showed that for a site of radius 1 μ the ratio k/D (the ratio of the velocity constant of the capture reaction to the diffusion coefficient of the PRP) should be greater than 10^6 cm^{-2}. A value greater than 10^8 cm^{-2} would give a good indication of the size of the site, and a value of 10^{10} cm^{-2} would provide excellent localization.

The numerical results considered above cannot be applied generally throughout the whole field of enzyme histochemistry. Holt pointed

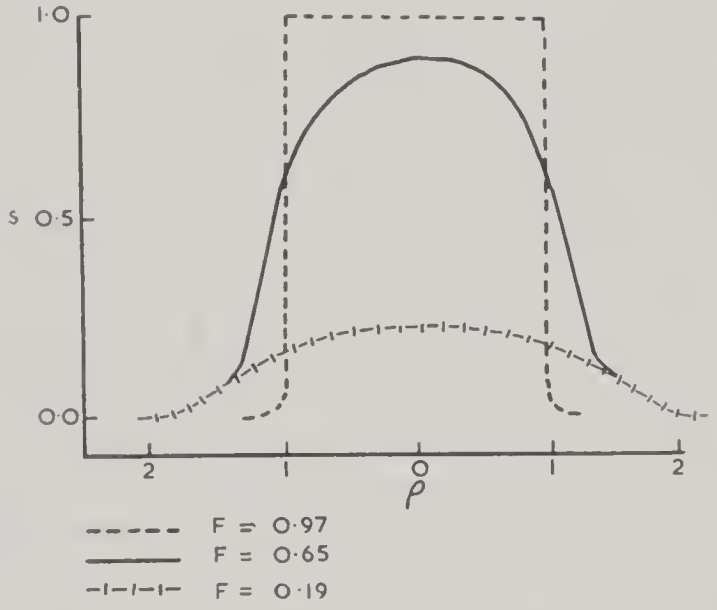

Fig. 101. Density distribution of FRP produced by a spherical enzyme site with different values for the localization factor. (After Holt and O'Sullivan.)

out that a number of things prevent this. The calcium phosphate techniques for alkaline phosphatase, for example, depend on the deposition of a PRP from a supersaturated solution and its absorption and/or crystallization on the tissues. Knowledge of the factors which influence the deposition of materials from supersaturated solutions is scanty and the effects of crystallization are difficult to assess and measure. Calcium phosphate precipitation in the alkaline phosphatase method has been considered from the theoretical point of view by Danielli (1958). The kinetics of chelation are also difficult to assess and satisfactory models have not yet been constructed for systems based on capture by metal chelation.

Post-incubation coupling

This type of reaction is based on the supposition that a PRP which is sufficiently insoluble will remain *in situ* without diffusion either

during incubation or during the subsequent performance of the necessary demonstrating reaction. The post-incubation coupling (post-coupling) principle has so far been applied only to the large group of azo dye methods. If the PRP is substantive as well as insoluble there is, of course, an even greater chance that the conditions outlined above will be satisfied. Nachlas *et al.* (1957) suggested that the post-incubation coupling methods have their greatest value where long incubation periods are necessary and when the pH optimum of the enzyme is in the acid range. Most diazonium salts in current use decompose on long standing in alkaline solutions and the resulting decomposition products stain the tissues diffusely, making interpretation of results a matter of some difficulty.

At acid pH levels the majority of salts couple poorly with naphthols and naphthylamines so that with simultaneous coupling methods diffusion of PRP becomes a serious factor. A further advantage of the post-coupling principle is that it avoids inhibition of enzyme by the diazonium salt and its stabilizer and diluent components. These are almost invariably present in commercially available diazotates.

The post-coupling principle has been applied to the histochemical localization of acid phosphatase (Rutenburg and Seligman, 1955), sulphatase (Rutenburg, Cohen and Seligman, 1952), β-glucuronidase (Seligman, Tsou, Rutenburg and Cohen, 1954), and glycosidases (Cohen *et al.*, 1952), using in each case esters or glycosides of 6-bromo- or 6-benzoyl-2-naphthol. Criticisms of the principle were put forward by Burton and Pearse (1952), by Pearse (1954), and by Defendi (1957). In particular the specific binding of naphthols, and especially substituted naphthols, to various tissue components has been considered to be one of the gravest objections to the post-coupling principle. Tests carried out by Defendi showed that 6-benzoyl and 6-bromo-2-naphthol exhibited not only the well-known naphthol binding to elastic tissue but also specific binding to various epithelial cells. Using fresh frozen (cryostat) sections, binding of naphthols by tissue components was observed to be dependent on the solubility and concentration of the naphthols, on the time of contact, the pH, and the type of tissue.

The more soluble naphthols (α- and β-naphthol) proved to have little affinity for any tissue component at alkaline pH levels though at an acid pH slight binding to liver cells occurred. The two insoluble naphthols had strong affinity for particular protein structures, especially the cytoplasm of parenchymal cells. Defendi concluded his paper with the observation that the post-coupling principle should be used with caution, not because of the occurrence of false positive results but because of the probability of false negative results.

Altered Solubility Methods

At the present time there are few methods making use of this principle in current use. It is based on the employment of coloured soluble substrates which are rendered insoluble when the solubilizing group is removed by enzyme aetivity. The insoluble dye is then presumed to precipitate at the site of enzyme activity.

A method for alkaline phosphatase making use of the altered solubility principle was described by Loveless and Danielli (1949) and a method for β-glucuronidase was produeed by Friedenwald and Beeker (1948). These methods are further considered in the appropriate chapters (XIV and XVII). It is unlikely that methods of this type will be developed on a wide scale, since only a small number of enzyme-sensitive solubilizing groups are known and in praetice the large substrate molecules produced are often hydrolysed with extreme diffieulty.

Intramolecular Rearrangement

This principle depends on the development of soluble substrates which, after hydrolysis, undergo molecular rearrangement to give highly coloured insoluble products. It has so far been employed, in preliminary explorations only, by Nachlas *et al.* (1957) for the development of a method for carboxylic acid esterases using an acetie aeid ester of indophenol. In this case the FRP was found to be too soluble to give accurate enzyme localization, but future studies in this direction may be expected to yield important results.

Development of Histochemical Techniques for Hydrolytic Enzymes

Properties of the Substrate. It follows, partieularly from the work of Holt and his assoeiates, that the first essential is that the substrate should be water-soluble and of low molecular weight. Unless these two criteria are satisfied the substrate will not diffuse rapidly enough into the tissues nor will it be present in sufficient concentration for the enzyme reaction to have zero order kinetics. Unless zero order kinetics are established the amount of FRP will not reflect accurately the level of enzyme activity.

Many of the substrates designed for use as esterase and phosphatase reagents are very insoluble in water and it becomes necessary to add organic solvents to the incubating medium in order to keep them in solution at all. If the PRP, as is often the case, is soluble in the ehosen organic solvent serious errors of diffusion may result. It is essential that the substrate be easily hydrolysed at the pH and temperature necessary for optimum enzyme activity. This attribute must not, of course, be aeeompanied by any tendeney to spontaneous hydrolysis. Many substrates in eurrent use (Naphthol AS esters, halogen-sub-

stituted indoxyl esters) are hydrolysed slowly, or with difficulty, and long incubation times are necessary. Even with fixed tissues this factor can lead to serious disturbances and with fresh tissues long incubations are entirely unacceptable.

A further necessary property which all substrates should possess is lack of polarity. Possession of polar groups may lead to attachment to specific protein components in the tissues and to interference with diffusion. Diffusion through lipid or lipid protein interfaces will also occur, and interference with this is a particularly serious factor in the case of enzyme systems which are enclosed in lipid or lipoprotein envelopes.

Properties of the Reaction Product. It is desirable that both the primary and final reaction products should not only be insoluble in water but also in lipid, since otherwise they may be concentrated in the latter. Such an occurrence is common with the reaction products of a large number of currently-used substrates, especially those in the azo-dye field. If the PRP is to be removed by a capture reaction it must be capable of reacting rapidly with the chosen reagent, but the question of substantivity (i.e. affinity for protein) of the reaction product is one which cannot easily be settled. Nachlas *et al.* (1957) take the view that substantivity for protein is a desirable property, but rightly stress that there should be no special affinity for *particular* protein components. Naphtholic reaction products of many current enzyme methods have a special affinity for elastic tissue. This is an undesirable quality. Strong polarity is also to be avoided, since this may lead to attachment of the reaction product to acidic or basic proteins at points distant from the site of enzyme activity. If the capture reaction is sufficiently rapid the effects of polarity will not be felt.

Modification of the substrate by the introduction of groups calculated to increase the reactivity of the PRP have been undertaken by Seligman and his associates. Further work on these lines cannot fail to be productive.

Properties of the Capture Reagent. There are several types of capture reagent in use in enzyme histochemistry. In the case of the indoxyl substrates the oxidizing agent (ferricyanide, cupric ions) plays this role, as do Ca^{2+} and Pb^{2+} in the older phosphatase techniques. In the coupling azo dye methods the diazonium salt is the capture reagent and with the metal chelation techniques for dehydrogenases it is the divalent cobalt or nickel ions of the chelator. There is thus no similarity between the various methods and few general rules can be formulated. Cotson and Holt (1958) showed that first order kinetics obtained when indoxyls were oxidized to indigoid dyes in the presence of excess O_2 at alkaline pH levels. It has not been shown that the other types of capture reaction employed in enzyme

histochemistry permit first-order kinetics, and it is probable that the diazonium salts usually employed do not do so. Nachlas *et al.* (1957, 1958) suggest appropriate modifications of diazonium salts which may be expected to increase the rate of coupling with naphthols and naphthylamines. They point out, however, that knowledge of the effect of various functional groups in the diazotate on its rate of coupling is at present extremely sparse.

Choice of diazotate for an individual coupling azo dye reaction is still largely based on empirical methods and the best-looking result is considered to indicate that the best diazotate has been selected. This is by no means necessarily true. Many diazotates strongly inhibit enzyme activity either by virtue of active groups in the molecule or because of the metal ions (Zn, Al, Mg, Cu) which are present as stabilizers. It has repeatedly been shown that partial inactivation of an active enzyme will lead to *apparently* better localization. This effect must never be overlooked and, if at all possible, it must be avoided.

Validity of Localization

With all histochemical enzyme methods, not only those for hydrolytic enzymes, the question of accurate localization has ultimately to be considered. In a few cases, where the enzyme is predominantly mitochondrial or microsomal, biochemical evidence is sufficiently strong to be considered accurate. In the majority of cases, however, there is no yardstick by which the validity of histochemical localization can be assessed.

The number of factors involved is considerable and it is almost impossible to take them all into account when designing or modifying histochemical methods for enzymes. The division of enzymes into diffusible or lyo, and fixed or desmo, forms was originally made by Willstätter and Rohdewald (1932). This conception has been studied experimentally by Nachlas, Prinn and Seligman (1956) and by Hannibal and Nachlas (1959). Many important points have been raised, and answered, and it is necessary to consider some of them here.

It is clear that diffusion of enzyme occurs to different degrees in different fixatives, and that it varies for each enzyme. Moreover, the loss of activity during fixation is due not only to inactivation by the chemical reaction of the fixative on the enzyme protein but also to diffusion of the lyo component into the fixative during fixation and into the medium during incubation. Measurements made by Nachlas and his associates show that in the absence of fixation 30 per cent. of esterase, 61 per cent. of alkaline phosphatase, 48 per cent. of acid phosphatase, 65 per cent. of leucine aminopeptidase and 5 per cent. of β-glucuronidase are present in the desmo form. After formalin fixation, as expected, an increase in the proportion of desmo to lyo enzyme takes

place in each case. Absolute acetone prevented significant loss of activity over the whole range of hydrolytic enzymes studied by Hannibal and Nachlas, but lower concentrations allowed the lyo component to diffuse. Lyo-esterase and lyo-alkaline phosphatase were observed to diffuse into absolute alcohol, but lyo-acid phosphatase and lyo-β-glucuronidase did not do so. Earlier studies by Doyle and Liebelt (1954) had already drawn attention to the diffusion of esterases into the incubating medium after fixation.

The procedures of fixation, and fixation and paraffin embedding, do not wholly prevent the diffusion of the lyo component of hydrolytic

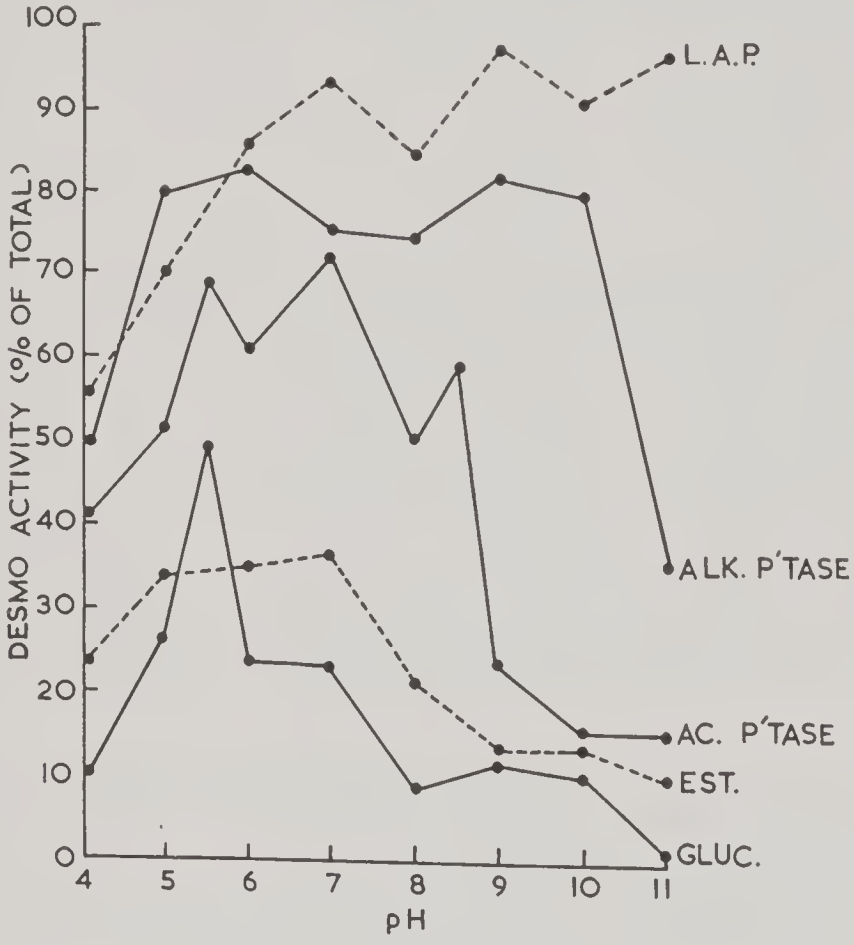

FIG. 102. Preservation of Enzymes as a Function of pH.

enzymes into the incubating medium. Many factors influence this post-fixation diffusion and among those which have to be considered are the duration of incubation, temperature, pH and the concentration of electrolytes and other additives.

Increasing the time of incubation, and elevation of the temperature, naturally cause increased loss of diffusible enzyme. Alteration of the pH of the medium produces very considerable changes in the amount of enzyme diffusion. Alkaline phosphatase and leucine aminopeptidase activities, in particular, are noted to be much higher at the pH optimum than in distilled water. The pH at which fixation is carried

out also exerts a strong effect on the amount of desmo enzyme subsequently recorded. The results obtained by Hannibal and Nachlas are shown in Fig. 102, on previous page.

Additives. Improved preservation of esterase can be obtained by the addition of sodium acetate or methyl cellulose and the activity of β-glucuronidase was observed by Hannibal and Nachlas to be doubled by addition of salts and 0·88M-sucrose, or methyl cellulose.

Chloral hydrate was employed by Fishman and Baker (1956) to preserve β-glucuronidase in thin tissue blocks. It was observed that in low concentration in dilute formaldehyde (0·1 per cent. in 6 per cent.) this substance improved the preservation of enzyme, as judged by the final results in tissue sections using the ferric-8-hydroxyquinoline method (Chapter XVII, p. 498). Further studies on the effect of chloral hydrate on the preservation of five hydrolytic enzymes have now been made by Baker, Hew and Fishman (1958). With their chloral-formalin fixative, β-glucuronidase activity was still present after fixation for over 3 weeks, while acid phosphatase was preserved for 6 weeks and esterase and lipase for 9 weeks. Alkaline phosphatase, on the other hand, was unable to survive for more than one day in chloral-formalin.

In their series of experiments referred to above Hannibal and Nachlas used chloral hydrate as an additive (1 per cent. in 10 per cent. formalin) and found that it did not prevent loss of the lyo component of any of the enzymes tested and that in some cases definite inactivation was produced. It is difficult to compare the results of Hannibal and Nachlas with those of Baker *et al.*, since not only did they employ different concentrations of chloral hydrate but also different terms of reference (percentage of desmo and lyo forms ; length of survival of enzyme).

Since the survival of alkaline phosphatase in chloral-formalin was enormously increased by buffering to pH 7·0 it is evident that chloral hydrate *per se* was not the important factor in this case, and the improved preservation of acid phosphatase and β-glucuronidase particularly might well be due to alteration of the pH of fixation so as to approach the value for optimum preservation of the lyo component (cf. Hannibal and Nachlas and Fig. 102).

Conclusion

It will obviously be necessary to consider all the methods, to be described hereafter in the enzyme section of this book, in the light of the theoretical and practical observations recorded in this chapter. Until such time as the necessary investigations have been carried out it is essential that those who use the methods in their present form should clearly understand their limitations, particularly in respect of enzyme localization.

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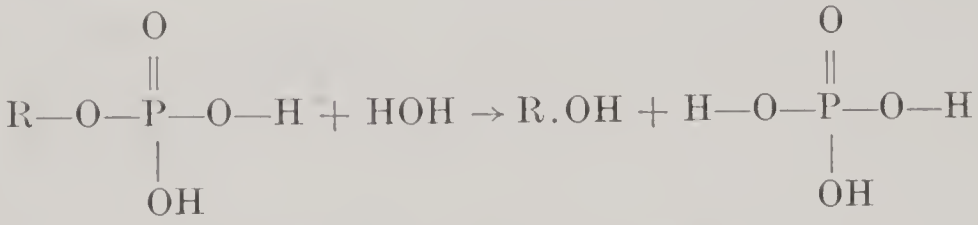
CHAPTER XIV

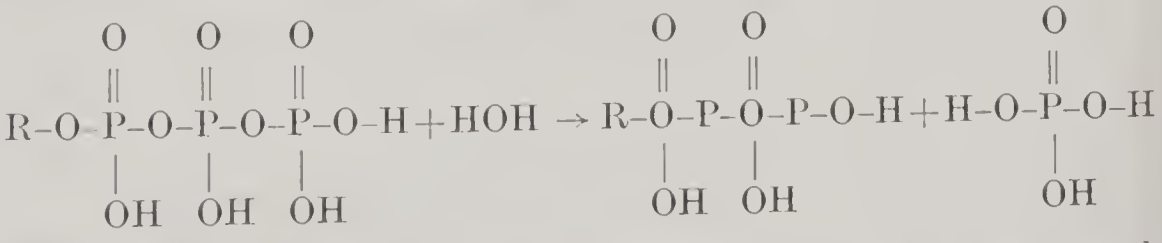
ALKALINE PHOSPHATASES

The Phosphatases

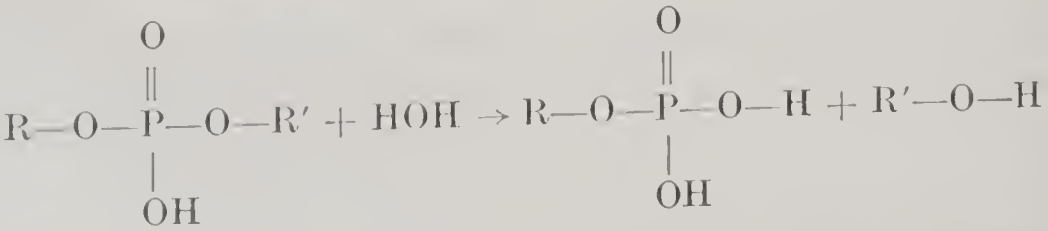
The hydrolytic enzymes responsible for the breakdown of phosphate esters can be divided into three types, the mono, di- and triphosphatases, and in histochemistry we are mainly concerned with the first of these. The phosphomonoesterases, generally speaking, are not specific in respect of the alcohol radical which is attached to the phosphoric acid group of the substrate and they will thus hydrolyse a wide variety of organic phosphates. In the equations given below, R is the alcohol radical.



In the case of phosphatases hydrolysing esters with three phosphate groups, of which adenosine triphosphatase is the chief example, the reaction occurs thus, producing a diphosphate :



Further enzyme action produces monophosphate and another molecule of phosphoric acid. This monophosphate is a substrate for the monoesterase and if the latter is also present the reaction proceeds to completion as in the first formula given above. In the case of the diesterases the reaction is essentially similar,



and again one of the reaction products is a substrate for the monoesterase. For this reason histochemical localization of the di- and

triphosphatases lacks precision unless it can be shown that the monoesterase is absent from the particular location concerned. Histochemical methods have been evolved for revealing the phosphomonoesterases which depend on the demonstration of either reaction product ; those depending on the deposition of the released phosphoric acid as calcium phosphate will be considered first, since up to 1950 all the important work on phosphatases was done by their use.

The phosphomonoesterases are divided on the basis of their optimum pH levels *in vitro* (cf. Kroon, Neumann and Krayenhoff Sloot, 1944). Those which work on the alkaline side of neutrality, and particularly at pH 9·0 and above, are called alkaline phosphatases, while those which work in the region of pH 5·0 constitute the acid phosphatases. The numerous curves with which Kroon *et al.* illustrated their paper show " summits " of two types, alkaline and acid. In none of the tissues examined could these authors find evidence of a third summit at a neutral pH.

It is probable that the most important *in vivo* activity of alkaline and acid phosphatases is concerned with the transfer of phosphate from one alcohol to another (Axelrod, 1948 ; Appleyard, 1948 ; Dixon, 1949 ; Meyerhof and Green, 1950 ; Dixon and Webb, 1953 ; Davison-Reynolds *et al.*, 1955 ; Morton, 1955 ; London and Hudson, 1955). This has been stressed by Danielli (1958) and by Dixon and Webb (1958).

$$R{-}O{-}\overset{\displaystyle O}{\overset{\|}{P}}{-}OH + R'OH \rightleftharpoons R'{-}O{-}\overset{\displaystyle O}{\overset{\|}{P}}{-}OH + ROH$$
$$\qquad\qquad\quad \underset{OH}{|} \qquad\qquad\qquad\qquad\quad \underset{OH}{|}$$

Since we have, at present, no histochemical methods for the detection of phosphokinases or transphosphorylases interpretation of alkaline or acid phosphatase activity as a manifestation of phosphate transfer must remain essentially speculative. The significance of much of the applied histochemistry of the phosphatases is nevertheless greatly improved if one assumes that high activity indicates increased phosphate transfer rather than hydrolysis of phosphate esters.

Techniques for the Non-specific Alkaline Phosphomonoesterases (DW. 106)*

Methods Depending on Deposition of $CaHPO_4$

Of all the histochemical techniques for hydrolytic enzymes the most important, historically speaking, is the method for alkaline

* These are the Dixon-Webb numbers of the enzyme or enzymes which the reactions to be described are designed to demonstrate.

phosphatase designed independently by Gomori (1939) and by Takamatsu (1939). Although the technique is simple to apply it is still necessary to emphasize the warning given by Danielli (1950) that from the point of view of the interpretation of results it is deceptively simple. The arguments and criticisms of the method which are given below will at least make this last point clear. A variation of the original technique (Gomori; 1946, 1952) is commonly used at the present time. It depends on the deposition of calcium phosphate at sites of enzyme activity when sections are incubated with an organic phosphate ester in the presence of calcium ions, usually at pH 9 or higher. Most of the phosphatases require magnesium ions as activators and these are therefore added in the form of small concentrations of magnesium sulphate or chloride. Mg^{2+} is the natural activator of most of the enzymes which act on phosphorylated substrates, especially the phosphokinases but not the phosphorylases. In many cases it can be replaced by Mn^{2+}. The mechanism of this activation remains uncertain. In some cases it is assumed that the metal forms an essential part of the active centre of the enzyme, and in other cases that it acts as a binding link between enzyme and substrate. It is also possible that the metal may directly activate the enzyme by altering the surface charge on the protein and thus its electrokinetic potential.

Various substrates are, or have been, employed, including α and β glycerophosphate, fructose diphosphate, yeast nucleic acid, yeast adenylic acid (adenosine-3-phosphate), phenyl phosphate, glucose-1-phosphate, creatine phosphate, thiamine pyrophosphate, barium phytate, adenosine triphosphate and muscle adenylic acid (adenosine-5-phosphate). In special studies like those of Gomori (1949a) many others were employed. The most usually employed substrate, sodium-β-glycerophosphate, is probably the best for ordinary purposes, being hydrolysed at a rapid rate which is exceeded, however, by that of phenyl phosphate and the naphthyl phosphates.

Following Gomori's example, tissues for phosphatase study have usually been fixed in three changes of absolute acetone at 4°, after which they have been dehydrated in absolute alcohol and embedded in paraffin wax. The double embedding process, using celloidin prior to embedding in wax, has also been employed with advantage. Although it is now realized (see section on fixation for enzyme studies in Chapter IV) that alkaline phosphatases are better preserved by other methods of fixation, the convenience of handling paraffin sections, prepared as above, and the fact that they can also be used for acid phosphatase, lipase and esterase methods, have ensured their practical survival. After incubation of the sections in a buffered solution of calcium nitrate, containing magnesium ions and substrate, it remains only to localize the precipitated calcium phosphate *in situ*. Two

principal methods have been employed for this. Gomori, and Takamatsu, originally used the von Kóssa silver stain but Gomori (1941) later used the cobalt sulphide procedure. Both are given in Appendix 14. Bourne (1943) considered the cobalt procedure to be the more efficient, but Kabat and Furth (1941) used von Kóssa's method and Newman *et al.* (1950) observed that if the cobalt method was used the control sections, incubated in calcium nitrate without substrate, showed a non-specific deposition of cobalt which subsequently appeared as cobalt sulphide. I agree with Gomori and Bourne in preferring the cobalt method for the final demonstration in most cases, since it produces sharper pictures with finer granulation. The von Kóssa procedure transforms the precipitated calcium phosphate into silver phosphate which is then reduced by the action of light to metallic silver. With some substrates (adenylic acid for instance), it may be preferable to the cobalt technique but the final appearances vary considerably. Where the enzyme is present in strength, the precipitate is usually dark brown and granular, but the final colour differs widely with different substrates. With some (fructose diphosphate) it is yellowish brown, while with others (muscle adenylic acid, thiamine pyrophosphate) it is black and often macro-crystalline. With substrates giving a macro-crystalline end result high power examination is fruitless. With both methods the presence of xylene in the final mounting medium must be avoided since it tends to cause fading, rapid in the case of cobalt, slower with the silver precipitates. Experiments conducted by Ruyter (1952) suggested that the cobalt sulphide method of conversion failed to demonstrate calcium phosphate when the concentration of the latter reached a certain critical level. Thus in the striated border of the duodenum, after a long incubation period, the deposits were well shown by the silver method but were unstained by cobalt sulphide.

Another method for demonstrating calcium phosphate precipitates, which I have not found to be efficient in practice, is the use of the dye sodium alizarin sulphonate which stains calcium phosphate deposits orange red. It was used by Bourne (1943), who added it to the incubating fluid. Many alternatives have been proposed but few have been found acceptable, even for critical studies. For instance, Bélanger (1951) suggested that sections should be examined by polarized light, in which the deposits show up clearly since the crystals of which they are composed are birefringent. Hancox and Nicholas (1956) used phase contrast microscopy in order to follow the reaction during incubation. They found the method more sensitive than the CoS technique. Takeuchi and Nogami (1954) devised a method of fluorescent staining and Molnar (1952) proposed the substitution of lead for cobalt and its subsequent staining with rhodizonate (see

Chapter XXIV, p. 701). Since the conversion of calcium to cobalt or to lead is not without its own peculiar problems (see below, p. 395) it would seem that fluorescent or visible staining of the deposits by lake formation with one of the more modern methods for calcium might well be preferable. The morin method (p. 935) and the copper phthalocyanin method (p. 933) are suggested for the purpose.

Factors Influencing the Result. In order that a calcium deposit may take place in the tissues the rate of production of phosphate ions must be high enough for the solubility product of calcium phosphate to be exceeded locally. The establishment of this condition is prevented by many factors :

Inactivation of the Enzyme during Fixation and Embedding. Fixation of enzymes, including the phosphatases, has already been considered in Chapters IV and XIII and will not be considered further here. The remaining processes connected with embedding have little effect on the amount of phosphatase remaining in the block, until we come to the stage of infiltration with paraffin. No loss is caused by dehydration in alcohol, and Mowry (1949) stated that, as clearing agents, light petroleum, xylene, benzene and chloroform were all satisfactory and appeared to cause no significant decrease in enzyme activity in 12–14 hours. Because of its suitability as a prelude to vacuum-embedding he expressed a preference for light petroleum. The usual methods of embedding in paraffin (M.P. 56°–58°) cause a significant but variable loss of enzyme which Danielli (1946) estimated at 75 per cent. The actual loss depends on both the duration and the temperature of embedding. If thin blocks of acetone or alcohol-fixed tissue are vacuum-embedded in low M.P. paraffin (42°–44°) enzyme activity is higher (Mowry, 1949), although it is much less than that observed in fresh frozen sections. For routine purposes it is sufficient to use ordinary paraffin provided that the duration of embedding is brief.

Inactivation during Mounting or Storage. Considerable loss of enzyme activity occurs if paraffin sections, mounted on egg-albuminized slides, are redried on hot plates at 60° or higher. On no account should temperatures higher than 37° be used for drying slides intended for use in enzyme histochemistry. Ruyter and Neumann (1949) drew attention to changes they considered to be due to diffusion of the enzyme through partial solubility in dilute ethanol. They claimed that transferring sections from 96 per cent. ethanol to water caused variation in the picture obtained and atypical localizations. These authors used a modified technique of placing 6 μ sections from doubly embedded blocks directly into the incubating medium, carrying out all further manœuvres with free floating sections and finally mounting on slides before removing the paraffin. In the ordinary course of

events little or no diffusion is caused by passage from xylene through the alcohols to water, provided that this is rapid. This procedure is best avoided altogether by placing sections from room temperature into light petroleum and thence, with a short rinse in absolute acetone and in water, into the substrate solution at 37°. If the section is allowed to dry before placing it in the incubating medium, small bubbles of air remain trapped in the tissue and the final effect is marred by these. With the Ruyter and Neumann technique the presence of paraffin in the sections raises the incubation time to something like 20 hours for rat kidney sections (1½ to 3 hours by the usual method). Strong objections must be made to it on this account and to any procedure which prolongs the incubation period (see below). Lison (1948) also drew attention to the possible solubility of phosphatases in watery solutions, suggesting that only the so-called desmo-enzymes would remain in tissue sections while the soluble lyo-enzymes would disappear during processing. In the same paper Lison stated that deterioration of phosphatase occurred with storage in the paraffin block but Gomori (1950b) could not confirm this. He maintained that no deterioration could be observed in sections cut from blocks stored at room temperature for 5 years. With Lison's warning in mind, I used at one time to store blocks for phosphatase studies at 4°, but I could observe no difference between sections cut from these and from similar blocks stored in the ordinary way.

Inactivation by Departure from Optimum pH. Raising or lowering the pH of the incubating medium from the optimum pH (for alkaline phosphatases pH 9·2–9·8) interferes with the result by slowing the rate of the reaction, although within fairly wide limits it does not inactivate the enzyme completely. Much more important is the effect of lowering the pH on the solubility of calcium phosphate. At pH 9·2 this compound is extremely, though not absolutely, insoluble and it has been considered to precipitate as soon as it is produced, thereby giving a picture which should correspond to the true localization of phosphatase (but see comparison with azo dye techniques below). At pH 7·5 it is much more soluble and will not precipitate locally unless the rate of production is very rapid. False localization is invariably seen when incubation is prolonged at levels below pH 8·5 and at pH 7·5 with even short periods of incubation. Apparent inactivation may be produced by pH changes which is in fact due to loss of lyo-phosphatase into the incubating medium (see Chapter XIII, p. 381). The effects of pH on alkaline phosphatase activity have been considered by Robbins (1950), Cacioppo *et al.* (1953) and Sonnenschein and Kopac (1953).

Effect of Activator. The principal activator for alkaline phosphatases is the magnesium ion whose accelerating effect was first noted

with a 30 per cent. solution would remove it from tissue sections entirely. They believed that by taking sections from xylene to water via various ethanol concentrations the true picture of localization of the enzyme was disturbed. This danger was certainly exaggerated. Newman *et al.* (1950) observed a diffuse precipitate distributed uniformly over the section and the glass slide, and thought that it was due to activity of enzyme which had diffused from the section into the incubating mixture. Other interpretations are obviously possible.

Diffusion of products of hydrolysis already briefly discussed under the heading of inactivation by departure from optimum pH, is the most important single factor not only in the alkaline phosphatase techniques but in many other techniques for the localization of enzymes. Four experiments were carried out by Gomori (1950b) in order to illustrate the importance of this factor. In the first, he showed that in sections completely inactivated by treatment with nitric acid, and then placed in a solution of purified alkaline phosphatase, an intense staining reaction could subsequently be obtained in which the distribution was entirely different from that seen in control sections. In the second, an active slide and an inactivated one were turned face to face, separated by a strip of celluloid at either end. Pairs such as these were incubated at various pH levels. Between pH 7 and 8·5 the inactivated tissue showed nuclear staining around centres of high activity. At pH 9·2 no diffusion was noted, although Jacoby and Martin (1949) had found evidence of diffusion even at this pH. Finally, inactivated sections were incubated together with active sections in a Coplin jar for 24–96 hours. Once again, between pH 7 and 8·5, nuclear staining occurred in the inactivated slides. These experiments confirmed the existence of both short and long range diffusion effects. Danielli (1946) carried out some similar experiments, studying the precipitation of $CaHPO_4$ by non-enzymatic procedures, the ability of $CaHPO_4$ to diffuse, and the sites of deposition of the alcoholic end of the phosphate ester (see below). He concluded that $CaHPO_4$ did not diffuse at pH 9 but that there was a strong tendency for it to deposit at the sites of so-called phosphatase activity. Gomori (1951a) found that no nuclear staining occurred in sections incubated with 0·01 M glycerophosphate at pH 9·3 for up to $2\frac{1}{2}$ hours, provided that the concentration of calcium ions exceeded 0·4 M. At lower concentrations of calcium nuclear staining became more and more apparent.

By means of a simple model Johansen and Linderstrøm-Lang (1951, 1952) investigated the liberation and diffusion of phosphate ions, and their precipitation by calcium ions as in the Gomori type of technique. These authors stressed the fact that not only must the solubility product of calcium phosphate be exceeded for a precipitate to occur locally but that " the essential condition for a local precipitation at the

site of enzymatic activity is therefore that the ion product at which spontaneous crystallization occurs is reached so rapidly that the entire cell is bathed in super-saturated calcium phosphate solution which will give rise to precipitation wherever this is induced, either by stray crystal nuclei embedded in the fixed cytoplasmic material or by other constituents of the cell." From their calculations Johansen and Linderstrøm-Lang concluded that spontaneous precipitation was unlikely to occur in the Gomori system, but that the precipitate was always induced either by preformed crystal nuclei or by other elements. What was measured, they considered, was just as much the distribution of precipitation centres as that of enzyme molecules.

As a possible mechanism of precipitation it was suggested that if calcium phosphate begins to precipitate somewhere in the neighbourhood of a site of enzyme activity (due to the presence of a preformed nucleus) then the HPO_4 concentration falls to a low value at this point. More phosphate ions therefore diffuse in but the diffusion is one-sided (more phosphate comes from the side of the site of production). Hence the solid particle grows asymmetrically and if growth on one side is accompanied by dissolution on the other side, the particle will wander towards the site of production of phosphate.

In opposition to these ideas Gomori and Benditt (1953) pointed out that several of the assumptions made by Linderstrøm-Lang and Holter were not valid for the conditions existing in tissue sections. In particular, they pointed out that the local concentration of enzyme in a cell might be 1,000 times the average concentration. Experimentally Gomori and Benditt observed that when phosphate was added to solutions similar to those used histochemically there was no tendency to supersaturation. They concluded that the factors contributing to false localization with the Ca-Co technique were not amenable to mathematical analysis and (Gomori, 1952) it was believed that most of the sources of error could be rectified by raising the concentration of calcium ions in the incubating medium to 0·05 or 0·1 M.

Johansen and Linderstrøm-Lang (1953) replied to these criticisms by comparing their work with that of Gomori and Benditt. It appears that in their calculations they assumed a *turnover number* (the number of moles of substrate reacting per minute per mole of enzyme) of 1,000, equal to 40,000 Bodansky units per gram. This is very different to the average figure of 700 units used by Gomori and Benditt. It is worthwhile to note at this point that the turnover number should be expressed in terms of active centres of enzyme (Dixon and Webb, 1958) and that the expression used above is better called the *catalytic constant*. In any case, unless the molecular weight and turnover number of alkaline phosphatase are known, theoretical calculations are necessarily arbitrary and probably inaccurate. According to Schramm

and Armbruster (1954), the molecular weight of alkaline phosphatase is about 60,000 and Morton (1957) has calculated the turnover number (catalytic constant) of intestinal alkaline phosphatase for phenyl phosphate at 38° as 236,000 moles per minute per mole of enzyme.

With regard to precipitation centres the observations of Danielli (1946) are still pertinent. He found that the artificial precipitation of calcium phosphate over a section of kidney resulted in a large amount of deposition in the nuclei and very little in the brush borders. Since an exactly opposite result occurs in the histochemical reaction Danielli considered that the quantitative distribution of precipitation nuclei did not affect the accurate localization of enzyme.

All the above considerations lead up to the difficult question of the staining of the nuclei by Gomori's method. We have to decide whether this is due to the presence of a phosphatase, specific or otherwise, or to diffusion artifact of one or the other variety.

Staining of the Nuclei and Nuclear Phosphatases. With the Gomori method for alkaline phosphatases it was consistently observed that the nuclei of cells in areas of intense staining were themselves deeply stained. For example, the nuclei of the proximal convoluted tubules of the kidney stained more darkly and in a shorter time than those of other portions of the nephron (see Fig. 103). This corresponded to the most intense phosphatase activity which was in the brush borders of the same tubules. Observations of this kind, and the experimental manœuvres considered above, suggested very strongly that the staining of the nuclei was due to diffusion artifact. While there can be no possible doubt that under a variety of conditions the staining of nuclei is due to this cause, we require to know whether it is invariably due to artifact or whether the nuclei in fact contain alkaline phosphatase, demonstrable by histochemical methods.

Newman *et al.* (1950), in a comprehensive study, used eleven different substrates and ten inhibitors and their results with respect to localization, substrate range, and inhibition effects suggested that the phosphate-liberating enzyme systems could be divided into three groups. **Group I enzymes** comprised the alkaline phosphatases, and these gave results with all substrates except the barium salt of inositol hexaphosphate. **Group II enzymes** were demonstrated when muscle adenylic acid or adenosine triphosphate (ATP) were used as substrates and they were regarded as specific 5-nucleotidases. (Although the latter do not hydrolyse ATP, this substrate is subject to spontaneous hydrolysis, one of the products being adenosine-5-phosphate.) **Group III enzymes** were only present in nuclei and their differentiation was based partly on the fact that with 5 of the 11 substrates nuclei were better shown than with the remainder and partly on the finding that heat, trichloracetic acid or M/4 to M/8 glycine or arginine inhibited the

staining of Group I sites but allowed the nuclei to stain as before. In order to show this so-called Group III activity, the authors incubated inactivated sections with the various substrates for no less than 7 days. If, under similar conditions, phosphate was released in the incubating fluid by any means whatsoever, the staining pattern was identical with that observed in the case of active sections. These results were probably due to adsorption of enzyme from the incubating medium, derived from active sections which were incubated at the same time.

Feigin, Wolf and Kabat (1950), using the techniques detailed above, determined that the increased nuclear staining adjacent to a locus of intense enzyme activity was due to enzymes of the same group as that locus and not to the so-called nuclear enzyme of Group III. They concluded that nuclear staining was an artifact " under some circumstances " and that it might be produced by diffusion of enzyme into the nuclei, after death, from sites in which it is normally present. That this is not the whole story is shown by the fact that nuclear artifact can be produced in sections freeze-dried less than a minute after death of the animal and subsequently fixed in absolute acetone for 30 minutes prior to staining. Continuing their studies, Feigin and Wolf (1953) compared the cobalt sulphide and silver nitrate methods for " developing " the final picture and reported that with the former nuclear staining might be both enzymatic and non-enzymatic. With the silver method only enzymatic staining was revealed, though this might be partly intrinsic and partly due to absorption of enzyme. This absorption of alkaline phosphatase by nuclei, as well as by other active sites, was demonstrated by Barter (1954), and also by Yokoyama, Stowell and Mathews (1951). More recently Feigin and Wolf (1954, 1957) were able to demonstrate, in cold acetone-fixed paraffin sections, a remarkable influence of magnesium ion concentration on the final localization of enzyme. If incubation was maintained at 5 minutes, there was only light cytoplasmic staining in the absence of magnesium, very considerable staining in the presence of 0·01 M-Mg, and practically none in the presence of 0·16 M-Mg. Nuclear staining, on the other hand, was present only when the highest concentration of magnesium was employed.

This nuclear activity bore no resemblance to other types of nuclear activity since it was observed in only a few organs, notably in guinea-pig endometrium, ovary and adrenal cortex. It was completely inhibited by 0·01 M-cyanide. The authors considered that their results indicated the presence of a true nuclear alkaline phosphatase.

Leduc and Dempsey (1951) studied the role of activation and diffusion in the alkaline phosphatase reaction. They showed that if an active section were incubated face to face with a heated section, no alkaline phosphatase reaction appeared in the latter and there was no

diffusion of calcium phosphate from the active section. Under the same conditions, nuclear staining was produced in a section inactivated by treatment with acid. The authors considered that this indicated a complete inactivation by heat but a reversible inactivation by acid, restored in this case by diffusion of an activator from the active section. I consider that their findings might equally well be due to physical differences in the affinity of the nuclear proteins for phosphate, brought about by heat or acid treatment. The fact that nuclear staining was still obtained in sections extracted with deoxyribonuclease or 0·1 N-HCl does not prove that affinity for the reaction product ($CaHPO_4$) was not the factor responsible, since the protein structure of the chromosomes remained intact.

With the evolution of histochemical techniques for revealing the alcoholic part of the phosphate ester, the existence of nuclear alkaline phosphatase has been further called into question. These techniques are fully considered in a succeeding section and only their bearing on the present question is dealt with here. The majority of the azo dye techniques for phosphatase depend on coupling of the reaction product with a diazonium salt present in the incubating medium, and these techniques are usually stated to show no activity in the nuclei. In fact, as already reported (Grogg and Pearse, 1952), the nuclei in acetone-fixed paraffin-embedded sections are often stained by coupling azo dye techniques, although in cold formalin-fixed frozen sections they are always colourless. Fig. 118 shows the staining of lymphocyte nuclei, and adventitial fibres of an artery, in rat lung. This observation indicates either the occurrence of enzyme diffusion during acetone fixation or the activation of phosphatase in occasional nuclei by precipitation of their proteins with acetone. In the azo dye method of Loveless and Danielli (1949) in which coupling was not used, the nuclear reaction was far more intense than that given by Gomori's method and the structure of the nuclei was in most cases " completely obliterated by deposits of dye." The significance of this remains uncertain, though Feigin and Wolf (1957) claimed that their experiments with the same substrate showed that non-enzymatic staining of nuclei by the phenol did not occur.

Lorch (1947) considered that the lack of nuclear staining with the coupling azo dye techniques was due to the insensitivity of the reaction when naphthyl phosphates were employed as substrates. Yokoyama, Stowell and Tsuboi (1950), comparing Gomori's method with the azo dye method of Manheimer and Seligman (1949), found that the former showed " definite nuclear activity " in many tissues and the latter " very little." Novikoff (1951) discussed the validity of the phosphatase methods for intracellular demonstration of enzyme. He stated that nuclear staining was absent with the azo dye method and con-

cluded that both this and the Gomori method were of little value for intracellular localization except in structures with extremely high activity. Gomori (1951a), using a newer modification of the azo dye method, also reported no nuclear staining in his preparations. Both these authors used acetone-fixed paraffin sections. On the other hand, Chèvremont and Firket (1953) invariably obtained nuclear staining in fibroblasts and myoblasts growing actively *in vitro*, by both the Ca—Co and azo dye techniques (Fig. 104, p. 400). These authors observed that procedures which interfered with the activity of the enzyme would produce negative results in the nuclei but that these could be restored by prolonging the incubation period.

The bulk of the experimental work detailed above suggests that the nuclear staining observed with the Gomori technique is mainly due to artifact. This conclusion is supported to some extent by observations on isolated nuclei from mouse or rat liver cells. Yokoyama *et al.* (1950) found little or no nuclear alkaline phosphatase activity in isolated mouse liver cells and Novikoff, Podber and Ryan (1950) found only a small fraction (15 per cent.) of the total enzyme activity in rat liver cells. Dounce (1950) also reported similar results. There remain, nevertheless, legitimate doubts concerning the interpretation of all nuclear staining with the calcium phosphate techniques as due to one or other type of artifact.

Histochemical Specificity of the Phosphatases. Much argument ranges about the point whether there are a number of different alkaline phosphatases or only one. Evidence derived from *in vitro* studies of resistance to inhibitors, and from studies on optimum pH, suggests that a number of alkaline phosphatases exist (Belfanti, Contardi and Ercoli, 1935 ; Bodansky, 1937 ; Gould, 1944). Other investigators (Glick and Fischer, 1945, 1946 ; Dempsey, 1949 ; Friedenwald and Maengwyn-Davies, 1950) stated that various alkaline phosphatases could be distinguished histochemically. Many authors, like Dempsey, Greep and Deane (1949), using various substrates, attributed the different pictures obtained to substrate specificity and referred, for instance, to fructose diphosphatase, nucleic acid phosphatase, etc. Gomori (1949a and b) studied the question of the unity or plurality of the phosphatases. He admitted, *ab initio*, that there could be no doubt that alkaline and acid phosphatases were distinctly different enzymes and that the individuality of the adenosinetriphosphatase of striped muscle, of pyrophosphatase, and hexosediphosphatase, was firmly established. Zorzoli and Stowell (1947) compared the distribution of glycerophosphatase and hexosediphosphatase in varying tissues. They believed that they could localize a specific fructose-diphosphatase by histochemical means. In order to determine whether the histochemical approach could give an answer to the question, Gomori (1949a)

used nineteen different substrates and found with eighteen of them no evidence, in acetone-fixed paraffin-embedded tissues, of any other than non-specific alkaline and acid phosphatases. With the nineteenth substrate, *p*-chloranilidophosphonic acid ($ClC_6H_4NHPO(OH)_2$), a different distribution was noted in the acid range which led him (1948b) to describe a specific phosphamidase (see p. 449). In further studies (1949b) he used ten additional substrates and observed intense reactions in 2 hours with all of these except the pyrophosphates, which required 24 hours' incubation. The distribution was identical with all substrates except 5-nucleotide. In this case, although in some organs the picture was identical with that produced by glycerophosphate, in others (brain, testis, smooth muscle, spleen) the difference was marked. Gomori considered that these findings indicated the presence of the specific 5-nucleotidase described by Reis (1937, 1940, 1951) and by Gulland and Jackson (1938). This specific 5-nucleotidase has also been demonstrated histochemically by Newman *et al.* (1950) and by Pearse and Reis (1952). In this last study, which is referred to below and illustrated in Figs. 114, 115, 116 and 117, two additional sites of strong 5-nucleotidase were noted, the adrenal medulla (rat) and pars nervosa of the pituitary (man). Although Gomori (1950a), in a single paper, unaccountably maintained that different alkaline phosphatases could not be demonstrated histochemically, the above evidence suggested that at least one enzyme could be so distinguished in the acid range and another two on the alkaline side.

The question of distinguishing substrate-specific phosphatases has been further considered by Novikoff (1958), who stresses the value of pH activity curves for various substrates when assessing the possibility of demonstrating specific phosphatases histochemically in a given tissue. He points out that in intestinal mucosa, for instance, the extremely high activity of non-specific alkaline phosphatase effectively prevents the demonstration of substrate-specific phosphatases such as 5-nucleotidase and adenosine triphosphatase. In other tissues, such as liver, the specific phosphatases can certainly be demonstrated histochemically (Wachstein and Meisel, 1957) owing to the small amount of alkaline phosphatase activity present. These points are further considered in the sections dealing with the substrate-specific phosphatases (pp. 412–425), and at the end of the section on non-specific alkaline phosphatase the important question of comparison between the calcium phosphate and the naphtholic substrate methods is dealt with. In a series of papers on the subject Bourne (1954a and b) and Baradi and Bourne (1951) have presented their findings on the hydrolysis of a large number of mono- and diphosphate esters by fixed tissue sections, using a Gomori type of procedure. Their substrates included dipotassium α-D-glucose-1-phosphate, barium D-fructose-1-phosphate, dipotassium D-galactose-

6-phosphate, barium L-sorbose-1-phosphate, tetrasodium 2-methyl-1,4-naphthohydroquinone diphosphate, tetrasodium 3,4′-dihydroxy-4-methoxychalcone diphosphate, riboflavin-5-phosphate and pyridoxal phosphate. Comparison was made in all cases with glycerophosphate.

Bourne considered that non-specific alkaline phosphatase was not responsible for the dephosphorylation of riboflavin and pyridoxal phosphates, since marked differences were evident in the localization of the calcium phosphate deposit in nearly all tissues examined. Differences between the various sugar phosphates and glycerophosphate were less striking, but Bourne concluded that his results indicated the existence of a "spectrum of phosphate-splitting enzymes with considerable overlap at pH 9." The results obtained by Baradi and Bourne (1951) in the tongue and olfactory mucosa of the rabbit also suggested to the authors the presence of a number of specific enzymes. This view was based largely on the differential inhibitions produced by vanillin, tea infusion, quinine, capsicum, aniseed, peppermint and coffee.

I examined the reaction of a number of the above substrates, together with some less likely ones, using fresh frozen cold microtome sections incubated in the acetone-containing medium of Fredricsson (1952, 1956) (Appendix 14, p. 869). Most of the substrates were hydrolysed only weakly by comparison with glycerophosphate and the principal and almost invariable difference was the presence of strong nuclear staining. This was completely lacking in glycerophosphate-incubated sections. In view of the number of alternative explanations for such findings, and in the complete absence of supporting biochemical data, Bourne's thesis must be considered unproven. If parallel biochemical and histochemical studies are carried out, strict comparison will only be obtained if the latter are done on fresh tissue (fresh frozen or freeze-dried sections). Fixation introduces too many variables into a problem already top-heavy with them.

Quantitative Estimation. Quantitative estimation of alkaline phosphatase in tissue sections was possible according to Gomori (1950b) only if laborious comparative experiments were carried out. Danielli (1950), on the other hand, suggested that incubation for a logarithmic series of times gave quantitative appreciation, the sites of highest activity appearing before the other sites. In all histochemical studies of the phosphatases, the shorter the incubation can be kept the more accurate is localization likely to be. An interesting method for the quantitative evaluation of Gomori-type histochemical preparations was evolved by Doyle (1950). This was based on the substitution of lead for cobalt (in the alkaline phosphatase method), the extraction of the final precipitate of lead sulphide with 5 N-HCl, and its conversion to methylene blue by the method of Fogo and Popowsky (1949). The

amount of methylene blue was measured spectrophotometrically at 670 mμ. With methods such as these (Doyle, Omoto and Doyle, 1951) only quantitation per section can be obtained. Radioactive tracer methods have been used by several workers following the early technique of Dalgaard (1948), who made use of ^{32}P-labelled glycerophosphate. This work has been extended by Barka *et al.* (1952), who employed ^{212}Pb, as lead nitrate, to precipitate the phosphate ions at pH 9·4. With the aid of a perforated lead disc, and a specially designed counter-tube, measurements could be made of the enzyme activity of relatively small areas of tissue. Further studies on these lines have been carried out by Shugar *et al.* (1957).

There is little doubt that the most convenient and easily applicable method is that of Barter *et al.* (1955) employing the interferometer microscope. This method affords only approximate values, however, since exact knowledge of the composition of the calcium phosphate deposit is not forthcoming. In calculating the mass of the deposit, therefore, an arbitrary value is employed instead of an exact one and, according to Danielli (1958) an error of ± 20 per cent. is possible.

Applications of the Gomori Method. Most of the work mentioned below was carried out by means of the calcium-cobalt or calcium-silver techniques. Now that they have been made more workable, the azo-dye techniques are being increasingly employed for demonstrating the non-specific alkaline phosphatases. There appear below references to the classical papers in phosphatase histochemistry with brief mention of a number of others which are of particular interest to pathologists.

The general distribution of alkaline phosphatase in normal tissues was described by Gomori (1941) and by Bourne (1943), and in normal and neoplastic tissues by Kabat and Furth (1941). The *kidney* has been investigated especially by Kritzler and Gutman (1941), Wilmer (1943, 1944), Bunting (1948), Eränkö and Niemi (1954), Eränkö and Lehto (1954), Wachstein (1955), Longley (1955), Sachs and Dulskas (1956) and by Tapia Freses *et al.* (1957), while the *endometrium* has been studied by Atkinson and Elftman (1946, 1947), Atkinson and Engle (1947), Arzac and Blanchet (1948), Atkinson and Gusberg (1948), Hall (1950), Erichsen (1953), Váczy *et al.* (1955) and by Skjerven (1956). The *ovaries* were studied by Corner (1944), and later by Leckie (1955), Ford and Hirschman (1955), Mukherjee and Banerjee (1955–56) and Dhom and Mende (1956). The *placenta* was studied by Hard (1946) and Dempsey and Wislocki (1947). The phosphatases of the *liver* were dealt with by Wachstein and Zak (1946a and b) and by Deane (1947), whose survey included also the *pancreas* and *salivary glands*. Later studies on the liver are those of Minamitani (1953), Meyner and Williams (1954), and Wajchenberg and Hoxter (1955). Jacoby and Martin (1951) compared the bio-

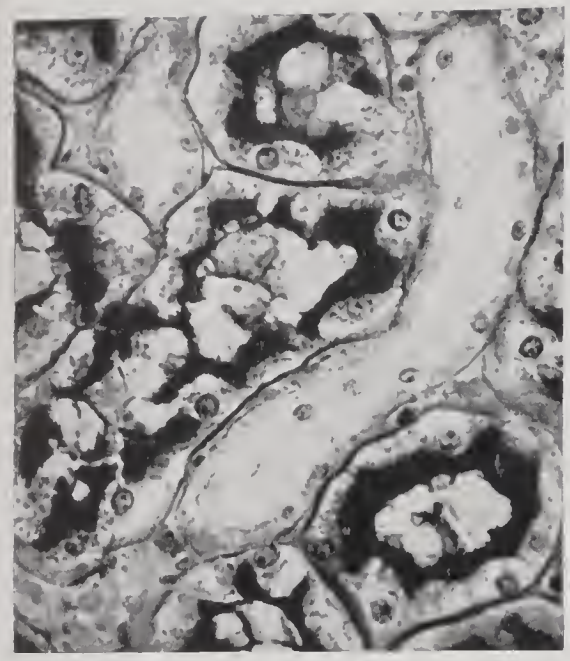


Fig. 103. Application of the Gomori Ca-Co method to a 5·5 μ paraffin section of rat kidney. Note staining of the brush borders of the primary convoluted tubules and of the nuclei. × 370.

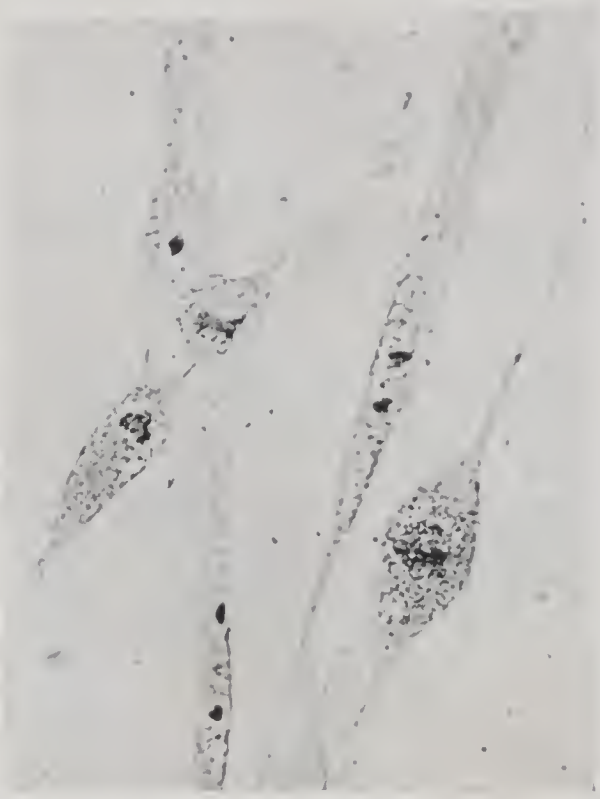


Fig. 104. Tissue culture (Chick embryo, muscle and connective tissue). Black stained regions were stained reddish-violet, grey areas orange-yellow. Coupling azo dye method for alkaline phosphatase. × 900.

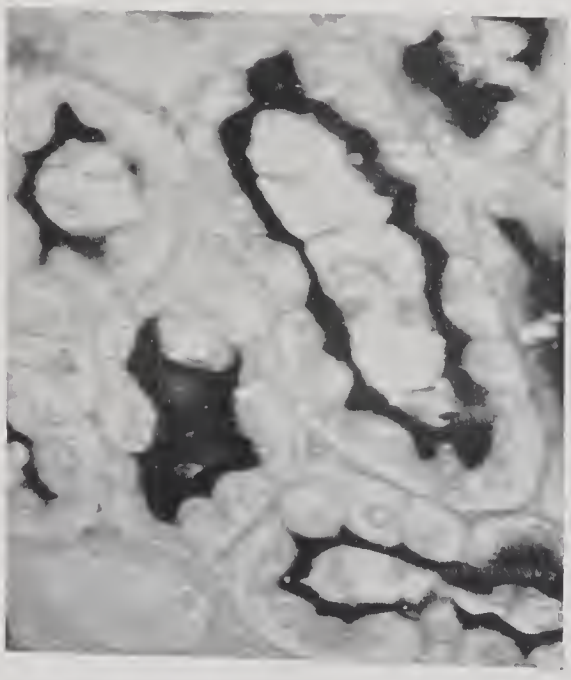


Fig. 105. For comparison with Fig. 103. Application of the coupling azo dye technique to the same material. Alkaline phosphatase is confined to the brush borders, the nuclei are unstained. × 400.

[*To face p.* 400.

FIG. 106. Rat. Agar granuloma. Alkaline phosphatase in leucocytes photographed after mounting in pure glycerine. Coupling azo dye method. × 750.

FIG. 107. As Fig. 106. Treated after incubation, and before mounting, with Lugol's iodine solution. Coupling azo dye method. × 750.

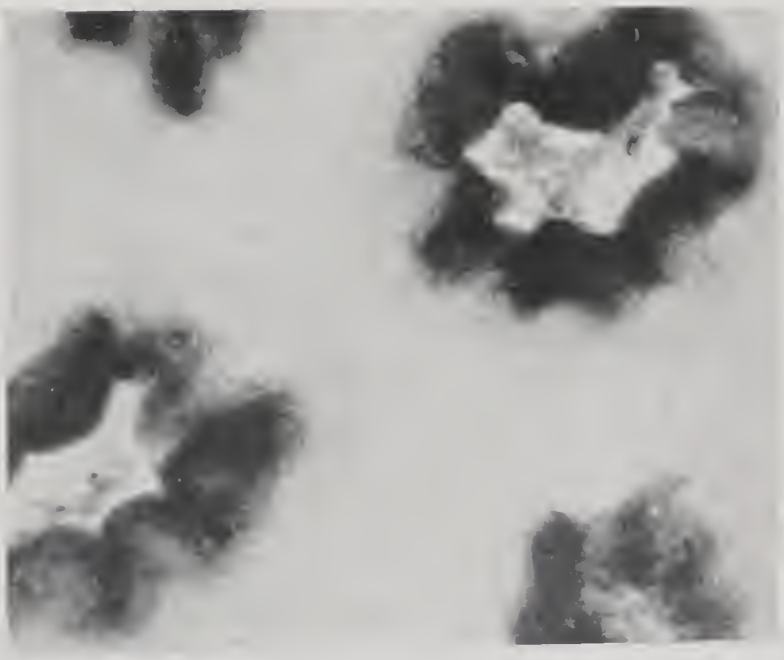


FIG. 108. Rat kidney. Formalin-fixed frozen section. Alkaline phosphatase in renal tubular cells. For comparison with Fig. 109. Standard coupling azo dye method (Salt 18). × 800.

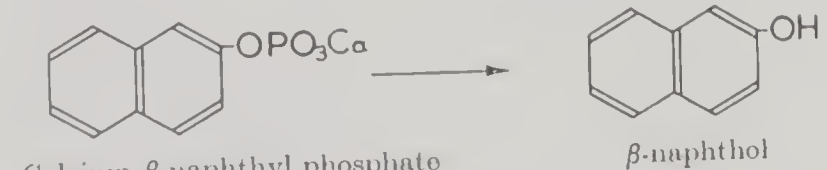
chemistry of the bile with the histochemical distribution of alkaline phosphatase in the liver, in the rabbit, dog and guinea-pig. There was a rough correlation between the bile alkaline phosphatase and the histochemical demonstration of the bile canaliculi. Krugelis (1946) and Emmel (1946) considered especially the intracellular distribution of alkaline phosphatase, while Danielli (1946) declared that, apart from the nuclei and brush borders of the kidney tubules, this enzyme seldom occurred in the cytoplasm except in rapidly regenerating tissue. He connected it especially with fibre formation, but Robertson, Dunihue and Novikoff (1950), repeating and extending the work of Fell and Danielli (1943) and of Danielli, Fell and Kodicek (1945), could find no such evidence. Many other authors have worked on this problem of the association of alkaline phosphatase with fibre formation (Johnson, Butcher and Bevelander, 1945 ; Bradfield, 1946 ; Jeener, 1947) and two more recent papers by Gold and Gould (1951) and Gould and Gold (1951) have supported the relationship between the two. These last authors claimed to have confirmed their histochemical findings with the Gomori method by using the azo-dye technique of Manheimer and Seligman (see below).

For other sites a short list is appended. *Blood and bone marrow:* Wachstein (1946), Storti (1951), Wiltshaw and Moloney (1955), Kaplow (1955), Cesàro and Gramata (1955–56), Boivin and Robineaux (1956), Graziadei and Zaccheo (1956), Trubowitz *et al.* (1957), Valentine *et al.* (1957). *Nervous system:* Landow, Kabat and Newman (1942), Sinden and Scharrer (1949), Shimizu (1950), Tolone and Ventra (1950), Naidoo and Pratt (1953), Chiquoine (1954), Feigin and Wolf (1955). *Skin and hair:* Johnson and Bevelander (1946), Fischer and Glick (1947), Foraker and Wingo (1955), Spier and Martin (1956), Kopf (1957). *Cartilage and bone:* Lorch (1947), Zorzoli (1948), Follis (1949, 1953), Majno and Rouiller (1951), Zorzoli and Nadel (1953), Schajowicz and Cabrini (1954). *Teeth:* Engel and Furata (1942), Bevelander and Johnson (1945, 1950), Greep, Fischer and Morse (1948). *Pituitary gland:* Abolins (1948), Abolins and Abolins (1949). *Thyroid:* McAlpine (1955), Haley *et al.* (1955). *Adrenal:* Verne and Herbert (1955), Allen and Slater (1956), Clayton and Hammant (1957) (Azo-dye method). *Nuclei and chromosomes:* Krugelis (1942), Danielli and Catcheside (1945), de Nicola (1949), Brachet and Jeener (1946, 1948), Sulkin and Gardner (1948), Firket (1952), Chèvremont and Firket (1953). *Cells in tissue culture:* Chèvremont and Firket (1949), Henrichsen (1956). The distribution and relationships of alkaline phosphatase and glycogen have been studied particularly by Horowitz (1942), Wislocki and Dempsey (1945), Johnson and Bevelander (1946) and Pritchard (1947), while Martin (1949), using rabbit liver, introduced a technique for demonstrating the presence of alkaline phosphatase and glycogen in the same section.

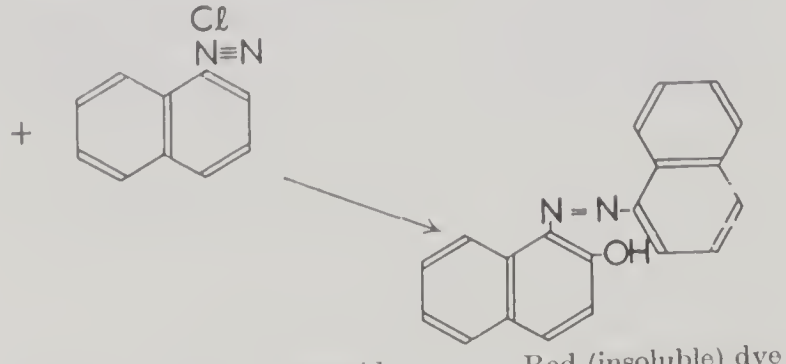
Simultaneous Coupling Azo Dye Methods (Unsubstituted Naphthols)

The first method for the histochemical demonstration of alkaline phosphatase by precipitation and demonstration of the alcoholic part of the phosphate ester used as substrate was described by Menten, Junge and Green (1944). This depended on the hydrolysis of calcium β-naphthylphosphate and the instant reaction *in situ* of the liberated β-naphthol with diazotized α-naphthylamine at pH 9·4 to give a red precipitate at the sites of phosphatase activity. Methods of this type are referred to as simultaneous coupling methods. The same principle was used by Danielli (1946), who used phenylphosphate as well as β-naphthyl phosphate to accomplish a similar reaction. Subsequently, modifications of the technique of Menten *et al.* were introduced by Manheimer and Seligman (1949) and by Gomori (1951a).

The essentials of the procedure, as detailed above, are the hydrolysis of a monoaryl phosphate, instead of an alkyl phosphate such as is usually employed in the Gomori technique, and the immediate coupling of the phenol, freed by hydrolysis, with a suitable diazotized amine to form an insoluble coloured precipitate at the site of enzymic activity. All subsequent simultaneous coupling azo dye methods, for other enzymes as well as for phosphatases, have employed this same principle. Such development was foreseen by the original authors. Menten *et al.* tried other phosphate esters before finally deciding on the use of β-naphthyl phosphate. Phenyl phosphates proved useless because the azo dye formed from the free phenol with a large number of diazotized amines was too soluble to be retained *in situ* in the tissues. β-naphthol, on the other hand, gave insoluble precipitates with a variety of amines, and of these α-naphthylamine was selected on account of the deep red colour and extreme insolubility of the resulting dye. The reactions involved are given below.



Calcium β-naphthyl phosphate — β-naphthol



α-naphthyl-diazonium chloride — Red (insoluble) dye

This ingenious method, performed on acetone-fixed paraffin sections, was technically laborious. After mixing the substrate and freshly diazotized α-naphthylamine the pH was adjusted to 9·4 with 6N NaOH and stabilized with veronal-acetate buffer. The brown precipitate which formed was removed by filtration and the solution cooled to 6°. Slides were incubated for 15–30 minutes, washed in water, counterstained with light green and mounted in glycerol. No activator, in the shape of the magnesium ion, was employed, and the optimum incubation time was 18 minutes at pH 9·4. The extreme rapidity of the reaction was notable and a deposit of the dye could be observed after 5 minutes' incubation of active tissue. This extreme rapidity, however, only exceeds the hydrolysis rate of glycerophosphate by a small margin. In active tissues the Gomori method gives a visible result in as short a time as this. Tests performed at 37° increased the rate of the reaction, but disintegration of the diazonium salt was also more rapid. Secondary products formed by the breakdown of the latter stained the background structures a diffuse yellow colour.

Modifications of the Original Method. The results obtained by Menten *et al.* were comparable with those of the Gomori method, but the authors did not mention the presence or absence of nuclear staining. Lorch (1947) stated that only sites of great enzyme activity could be demonstrated by this method and she attributed the observed absence of nuclear staining to the weakness of these sites in phosphatase. One of the outstanding drawbacks to the method as outlined above was the necessity for the preparation of a fresh diazonium salt for each batch of the incubating medium. Manheimer and Seligman (1949) overcame this by forming a stable diazotate of α-naphthylamine by the addition of naphthalene-1 : 5-disulphonic acid. They also used commercially available diazotates of *o*-dianisidine and 2-amino-4-chloroanisole (see Table in Appendix 14, p. 870). Using acetone-fixed paraffin sections and a pH of 9·4, deposition of dye in phosphatase-rich tissues was noted within 1 minute. By using the diazonium salt in low concentration, together with low temperature and short incubation, the brownish staining of the background was reduced to a minimum. Because of the solubility of the azo dye in organic solvents, the final mounting was made in an aqueous medium. Manheimer and Seligman used calcium α-naphthyl phosphate as well as the β-naphthyl salt, but found that it was unsuitable as a substrate for alkaline phosphatase because it gave relatively weak staining under optimal conditions. These authors compared their modified Menten-Junge-Green method with Gomori's calcium-cobalt method, in a wide variety of tissues, and reported a close correspondence. No specific study of the reaction of the nuclei was made, but it was noted that " some staining occurred in tumour-cell nuclei." The advantages of the method were considered

to be: (1) The incubation period was short and hence the method more sensitive. (2) Control sections were unnecessary as naphthols are not normally present in the tissues. (3) The azo dye was very much less soluble than $CaHPO_4$, so that pictures were very much sharper and diffusion artifact was reduced. (4) Development of colour could be watched, and stopped at the optimal time.

Theoretical and Practical Considerations. A further modification of the Menten-Junge-Green method for alkaline phosphatase was suggested by Friedman and Seligman (1950). This involved the substitution of the relatively insoluble calcium salt by the readily soluble sodium salt. This modification was used by Gomori (1951a), who incubated acetone-fixed paraffin sections with 0·002 to 0·005 M-sodium α-naphthyl phosphate at pH 9·4 in the presence of a variety of stable diazonium salts. The buffered substrate-diazotate medium was filtered into a Coplin jar and the sections incubated at room temperature. Within a few minutes the sites of strong phosphatase activity were observed to be coloured black, blue or red, according to the diazotate employed. After 15 minutes or so the incubating medium became dark and turbid and the author advised its renewal at this stage. The background staining, due to breakdown products of the diazotates, was easily removed with 1 per cent. acid alcohol according to the author. Gomori (1951b) did not consider the reaction to be faster than that obtained in comparable sections by means of the Ca-Co technique. A comparison between his two methods, applied to acetone-fixed paraffin sections of rat kidney, is given by Figs. 103 and 105. Absence of nuclear staining with the azo dye method is the most important point to observe.

The superiority of the Gomori modification is due to two things; the substitution of the far more water soluble sodium salt, which enables the optimum concentration of substrate to be employed, and the substitution of the α-naphthyl salt. The azo dyes produced by coupling α-naphthol with diazonium salts are far more insoluble in water than those produced with β-naphthol and diffusion of the final reaction product is thus diminished. Grogg and Pearse (1952) reported attempts to improve the coupling azo dye technique for alkaline phosphatase still further, and subsequent work on these lines is discussed below.

Sodium α-naphthyl phosphate has been confirmed as the substrate of choice and it has been observed *in vitro* (King, 1952) that the hydrolysis of α-naphthyl phosphate proceeds as rapidly as that of phenylphosphate at alkaline pH levels. Since it is well known that acetone fixation and paraffin embedding destroy at least 70 per cent. of alkaline phosphatase present in the tissues, it is better to use short fixation in cold alkaline formalin (4°, 8–16 hours) and frozen sections.

Alternatively, use may be made of the principle of simultaneous fixation, applied to freeze-dried or cold microtome sections. In sections of these types preservation of enzyme is good, fixation artifact small and enzyme diffusion also small, so that the most important single factor in the performance of the method becomes the choice of a suitable diazonium salt for coupling at an alkaline pH.

Certain properties of diazonium salts have particular bearing on the problem. Inhibition of enzyme activity is produced by all diazonium salts in varying degree, and the possibility of reducing this inhibition by lowering their concentration is prevented by the fact that for efficient coupling with the reaction product (α-naphthol) a large excess of diazonium salt is necessary. The optimum concentration for most diazotates was found to be in the region of 1 mg./ml. of substrate solution. If the concentration was much lower than this (0·1–0·2 mg./ml.) two particular effects were noted. First, diffusion of α-naphthol occurred, and, secondly, the final colour of the azo dye precipitate was less strong. With diazonium salts like Fast blue B, which gives a black final product at the optimum concentration, for instance, the resulting dye with lower concentrations was invariably red. If the concentration of diazonium salt was higher than 5 mg./ml. inhibition was very great and no staining at all resulted except in situations of strong enzyme activity. The effect of the zinc or aluminium salts, present in most stable diazotates, on the activation or inactivation of alkaline phosphatase has not apparently been considered. The optimum concentration of $ZnCl_2$ for activation has been given as 0·001 M (0·0136 g./litre) and the average concentration of $ZnCl_2$ in the diazotates is 5 per cent. At a concentration of 1 mg./ml. of the diazotate 0·05 g./litre of $ZnCl_2$ will be present, a figure about $3\frac{1}{2}$ times greater than the optimum concentration for activation. The latter is unfortunately rather close to the figure for minimum concentration of diazotate for effective coupling.

A second property of the diazonium salts which is of great importance is the non-specific staining of the tissues resulting from their breakdown products. In extreme cases this may be mistaken for a positive reaction due to enzyme activity, and it is not always removable beyond a certain point. It is essential that the colour of the azo dye final product should be as dark as possible and that the size of the precipitate should be small. The first increases the sensitivity of the method by raising the point of minimum appreciation, the second enables accurate localization to be made on the intracellular level. Since the azo-α-naphthol dyes, and to a greater extent the azo-β-naphthol dyes, are soluble in lipids, the final product precipitated in the tissues should have particles visible under high magnification. If the final product is not particulate, staining of lipid components of the

tissues is likely to be indistinguishable, if it occurs, from the specific deposition of dye.

Unpublished observations by Loustalot (1955) suggested that some of the changes occurring after storage in preparations made by simultaneous coupling azo dye techniques for alkaline phosphatase might be due to continued enzyme activity on residual substrate. He found considerable improvement after brief treatment with Lugol's iodine (Figs. 106 and 107, p. 401). There is no doubt that this procedure can reduce storage artifacts, but the mechanism by which it does so is far from clear.

I have tested a number of stable diazotates, thought likely to be suitable on theoretical grounds, as coupling agents in the alkaline phosphatase method. In each case a note was made of (1) the speed of the reaction, (2) the rate of decomposition of the diazonium salt (and hence the amount of non-specific staining), (3) the colour of the final product, and (4) the type of precipitate. The results, in the case of fifteen stable diazotates, appear in Table 30. A very thorough survey, on these lines, has also been carried out by Goessner (1958). His findings, which agree substantially with my own, are referred to more fully in succeeding paragraphs.

The question of diffusion of the reaction product was controlled by incubating two sections face to face, one inactivated by heat (15 minutes/90°) and Lugol's iodine solution (15 minutes), the other untreated. No diffusion of the reaction product was detected with any of the salts within the limits (100 μ) of the distance between the two sections. Absence of enzyme inhibition by salts 2, 6, 7, 9, 13 and 15 to 22, at the optimum concentration, was indicated by the production of a strongly positive result in 2–5 minutes in sections of rat kidney. This is shown in the case of salt 9 in Plate IVA, p. 436. With all the other salts mentioned in the table inhibition was considerable. The decomposition of salts 9, 20, 21 and 22 was negligible, even after incubation for 8–16 hours ; with salt 2 it was slight up to 4 hours, and with salts 7 and 16 it was slight up to 1 hour. Salt 6, on the other hand, rapidly stained the tissues dark brown and salt 14 equally rapidly stained them dark red. Precipitate size was largest with 2, smaller with 7, 9, 20 and 22, and very much smaller with 6. The reactions with salts 2, 9, 20 and 22 were the best obtained in respect of speed, colour and lack of decomposition. The full method, for application to both acetone-fixed paraffin sections and to formalin-fixed frozen sections, is given in Appendix 14, p. 872.

The investigations carried out by Goessner (1958) on the choice of diazonium salt in the simultaneous coupling method, using α-naphthyl phosphate, were done with formalin-fixed frozen sections. He recommended three salts particularly for the purpose : Echtrotsalz

TABLE 30

Properties of Coupling Agents at pH 9·2
(Frozen Sections)

	Diazonium salt of	Inhibition	Decomposition	Colour	Diffuse or particulate
1	1-amino-anthraquinone	+++	+++	Brick red	Diffuse
2	4-benzoylamino-2 : 5-dimethoxy-aniline	+	+	Black	Particulate
3	*p*-nitroaniline	+++	++	Brick-red	Particulate
4	4-chloro-2-nitroaniline	++++	+	Rose-red	Particulate
5	2 : 5-dichloroaniline	++	++	Red-brown	Particulate +
6	*o*-dianisidine	+	++++	Black	Diffuse
7	4-chloro-*o*-anisidine	+	++	Brick-red	Particulate
8	5-nitro-*o*-anisidine	++	+++	Red-brown	Diffuse
9	5-chloro-*o*-toluidine	+	Nil to +	Brown	Particulate
10	4-nitro-*o*-anisidine	+++	+++	Brown-red	Particulate
11	3-nitro-*p*-toluidine	++++	++	Brown-red	Particulate
12	3-nitro-*p*-anisidine	++	++	Brown-red	Particulate +
13	4-amino-3 : 1′-dimethyl azobenzene	+	+++	Dark brown	Particulate
14	4-amino-2 : 5-dimethoxy-4-nitroazobenzene	+++	++++	Purplish-black	Particulate +
15	4-amino-4′-methoxy diphenylamine	++	++	Orange-red	Particulate
16	3-nitro-*o*-toluidine	++	+	Brown	Particulate +
17	4-amino-4′-nitro-3 : 6-dimethoxy azobenzene	+++	++	Red-brown	Particulate +
18	4-amino-3 : 1′-dimethyl-azobenzene	+	+	Brown	Particulate
19	2-methoxy-5-diethylamino-sulphaniline	++	Nil to +	Red-brown	Diffuse
20	2-benzoylamino-4-methoxy-toluidine	+	Nil to +	Brown-black	Particulate
21	4-amino-diphenylamine	+	+++	Black	Diffuse
22	*p*-*p*′-diaminodiphenylamine	+	Nil to +	Black	Particulate

In the above Table degrees of inhibition are registered from + to ++++, the former indicating almost complete absence. Similarly, in the case of decomposition, one + means practically no visible staining of the background structures. The reference particulate means that the dye particles were large enough to be clearly visible with the 2/3rds objective ; diffuse means a particle size not appreciable at this magnification. Numbers given are Laboratory Reference Numbers given in the Table of Diazotates (Appendix 14, p. 870). Numbers in bold type are those of recommended salts. Apparently identical salts have different stabilizers.

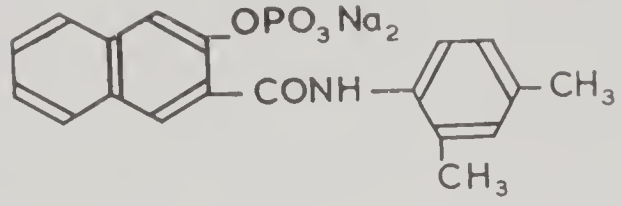
TR (9), Echtblausalz BB, and Variaminblausalz B (21). His average incubation time was 30–90 minutes, at 22°, in 0·1 M-phosphate at pH 9·2. The final choice of diazonium salt must still, unfortunately, be governed by the results, and these will depend especially on the type of tissue preparation employed. In the case of alkaline phosphatase, a predominantly microsomal enzyme with a large lyo-component, we can expect difficulty in obtaining precise intracellular localization. The azo dye methods with simple naphthol phosphates in fact give only rough intracellular localization of enzyme. They may be improved by faster coupling, by the use of protective media and,

perhaps, by the use of simultaneous fixation. They are particularly applicable to fresh and to formalin-fixed frozen sections.

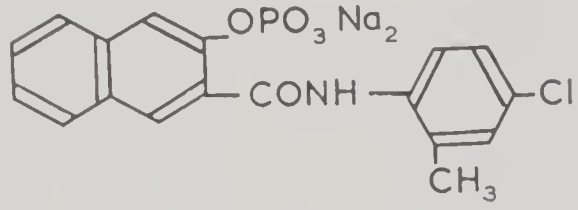
Simultaneous Coupling Azo Dye Methods (Substituted Naphthols)

The main work on the use of substituted naphthol phosphates in simultaneous coupling techniques for alkaline phosphatases is that of Burstone (1958a and b) though similar work with a single substitute (Naphthol AS phosphate) has been reported by Rutenburg *et al.* (1958). The phosphate esters of a variety of complex arylides of 2-hydroxy-3-naphthoic acid were prepared by Burstone using modifications of the technique of Freeman and Colver (1938). The most suitable esters for use as substrates for non-specific alkaline phosphatase were those of Naphthol AS-MX (I), AS-TR (II) and AS-B.I (III), whose formulæ are given below :

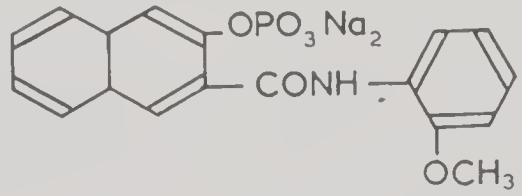
I



II



III



These substrates were used as saturated solutions in buffered 2 per cent. dimethylformamide at pH 8·3. The usual incubation period for paraffin sections was 15–30 minutes and good coupling was obtained with Fast blue RR (2), Fast red RC (7), Fast red TR (9), Fast red RL (16), and Fast violet B (20). Red violet LB (5-benzamido-4-chloro-*o*-toluidine) and Blue BBN (4-amino-2,5-diethoxy benzaniline) were also found to be successful, but the latter had a rapid rate of decomposition. Although Burstone employed many types of tissue preparation his best results were obtained with freeze-dried sections. These were incubated without subsequent fixation. The results he obtained were " essentially similar " to those observed with the older phosphatase methods, but localization was claimed in all cases to be sharper and the

colours more intense. There is no doubt (see Figs. 108 and 109, pp. 401 and 410) that the localization of the final reaction product is particularly clean and precise. What remains uncertain is the relation of this to the total non-specific alkaline phosphatase activity of the section.

Apart from their application to freeze-dried tissues the Naphthol AS methods are particularly applicable to blood smears (Monis and Rutenburg, 1958) for the demonstration of leucocyte phosphatases. A combination should be chosen which allows a stable stock solution to be prepared (e.g. AS-MX phosphate) and which produces a dark blue final reaction product (e.g. Fast blue BBN). In the case of fresh frozen and formalin-fixed frozen sections the results obtained with the Naphthol AS-phosphates do not justify their employment in place of the more usual α-naphthyl phosphate.

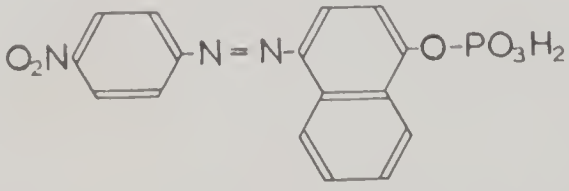
The precise localizations obtained with the AS-phosphates are a reflection of the low solubility of their naphtholic reaction products and of the affinity of these for protein (substantivity). Furthermore, they are highly stable esters and can be kept for long periods at alkaline pH levels in the form of stock solutions. These are undoubted advantages. On the other side, however, the AS-phosphates possess certain undesirable qualities. First, their solubility in aqueous media is low and the optimum substrate concentration is therefore unobtainable. Secondly, they are larger molecules than the simple naphthols and possess a variety of polar groups. These factors will tend to bring the concentration of substrate at the enzyme site to a very low level. Burstone's observation that the pH optimum of alkaline phosphatase with his substrates is in the region of pH 8·3 (cf. the α-naphthol method at pH 10) supports the finding of Neumann (1949) that the pH optimum of alkaline phosphatase is dependent on substrate concentration.

A third factor of importance is the low coupling rate of hydroxynapthoic anilides. Defendi and Pearse (1955) found that the average half-time of the coupling rate with Fast blue B and a variety of these anilides was 62 seconds compared with 300 millisecs for α-naphthol and 1,800 millisecs for β-naphthol, at an alkaline pH level. Because of the low solubility and high substantivity of hydroxynaphthoic anilides, however, the importance of this factor is considerably reduced. Until critical studies have been made on the Naphthol AS-phosphate methods (and on the Naphthol phosphate methods for that matter) there will continue to be doubts that sharpness of localization is obtained at the expense of sensitivity. That is to say, some sites of enzyme activity will not be recorded. Comparative studies of the spectrum of phosphatases by Naphthol and Naphthol AS methods, carried out by the zymogram technique of Hunter and Markert (1957), would seem to be indicated. Using Naphthol AS-acetate, these authors found that

this substrate was hydrolysed by only a proportion of the total number of esterases shown by α-naphthyl butyrate. It is possible, as suggested by Burstone in the case of bone, that an entirely new enzyme is being demonstrated. Biochemical confirmation of such a possibility is necessary before it can be completely accepted.

Non-coupling Azo Dye Methods

A method was devised by Loveless and Danielli (1949) which was designed to overcome the disadvantages of the Menten-Junge-Green technique. According to the authors these disadvantages were : (1) the instability of diazonium salts and their fresh preparation ; (2) the necessity for low temperatures, thus denying the enzyme its optimal conditions ; and (3) the inhibitory effect of diazonium salts. It will be realized that the first of these has been removed by the use of stable diazotates and the second, at least partially, by the successful employment of temperatures as high as 37°. The third is still a significant factor, though it can be reduced to a low value by using a suitable diazotate. Loveless and Danielli produced a coloured phosphate ester which, on hydrolysis, yielded an insoluble highly coloured base which had no tendency to crystallize in the tissues but remained amorphous. This ester, *p*-nitrobenzene-azo-4-naphthol-1-phosphate



was moderately soluble and stable in the absence of the enzyme. It had no inhibitory effects and was hydrolysed rapidly. With such properties the substrate should have been ideal for the purpose for which it was designed. Using the sodium salt, the results were studied with sections of rat kidney fixed in 80 per cent. alcohol and embedded in paraffin. By comparison with the glycerophosphate method it was seen that the dye was deposited in all the usual sites but, in addition, the nuclear reaction was far more intense. It was possible to obtain permanent sections mounted in balsam, provided that clearing in xylene was carried out rapidly, but this theoretical advantage over the coupling techniques has been offset by the application to these of diazotized *o*-dianisidine as coupling agent. The azo dye, formed by the union of this salt and α-naphthol is insoluble in alcohol and in xylene, though not in mixtures of the two, so that permanent mounts can be obtained if required.

Although the Danielli method is of considerable theoretical interest, especially in relation to non-coupling azo dye techniques which have

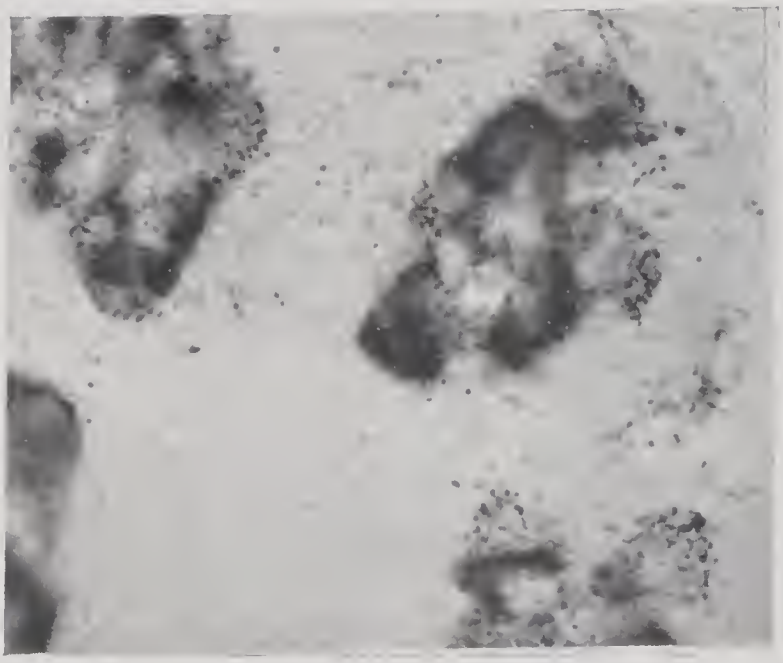


Fig. 109. As Fig. 108. Burstone's Naphthol AS-MX method. × 800.

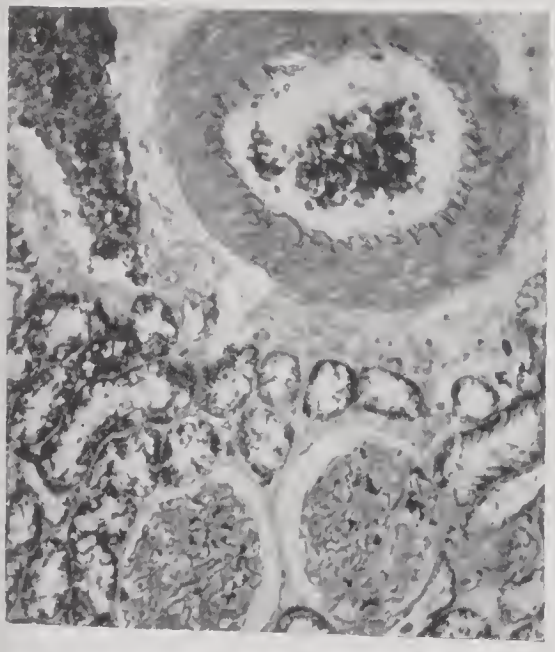


Fig. 110. Rat renal cortex. Disposition of 6-bromo-2-naphthol released enzymically by alkaline phosphatase at pH 9·2. Coupled with Fast Blue B salt at pH 7·5. Note diffuse tubular staining and staining of red cells, artery wall and elastica. × 145.

Fig. 111. Rat renal cortex. Disposition of alkaline phosphatase shown by a simultaneous coupling azo dye method. Only the primary convoluted tubules and the arterial adventitia are stained. × 145.

[*To face p.* 410.

been developed for other enzymes, its disadvantages are too great for it to be of practical use. In spite of the authors' statements, it is evident that the free base was to some extent soluble in watery solutions and that diffusion of the dye to neighbouring structures occurred. It is unlikely that diffusion of phosphatase to the nuclei could have taken place (in 80 per cent. alcohol) in sufficient amount to cause the observed result. The great advantage of the coupling over the non-coupling azo dye techniques, which outweighs any of the disadvantages pointed out by Loveless and Danielli, is that provided that the rate of production of naphthol is not too rapid for almost instantaneous coupling to occur, an entirely water insoluble product is deposited at the sites of enzymic activity. Since the localization of alkaline phosphatase by the non-coupling technique is probably inaccurate, the method is unlikely to find employment in modern histochemistry.

Post-coupling Azo Dye Techniques

Because of the rapid coupling obtainable in simultaneous coupling techniques with α- and β-naphthols, and the relatively rapid coupling obtainable with the substituted naphthols, post-coupling techniques have scarcely been used for the demonstration of alkaline phosphatases. The post-coupling technique of Danielli (1946), using sodium phenolphthalein phosphate, is of historical interest only.

The primary reaction product of many of the existing post-coupling methods for hydrolytic enzymes is 6-bromo-2-naphthol. Its phosphate can be used as a substrate for alkaline phosphatase in both post-coupling and simultaneous coupling techniques. The results obtained are shown in Figs. 110 and 111, p. 410. It is obvious that at least at alkaline pH levels the solubility of 6-bromo-2-naphthol is sufficient to allow diffusion to occur with resulting non-specific staining of many tissue components (see Pearse, 1954 ; Defendi, 1957).

Comparison between Ca-Co and Naphtholic Methods

Using 10 μ fresh frozen sections Burgos *et al.* (1954, 1955) compared in a variety of tissues the apparent localization of alkaline phosphatase afforded by the Ca-Co method (glycerophosphate, fructose diphosphate) and by a simultaneous coupling method (α- and β-naphthyl phosphates). In both cases their media contained 0·2 per cent $CaCl_2$ and 0·01 per cent. $MgSO_4$, and the validity of the azo dye method was checked by omitting the diazonium salt and by the subsequent demonstration of the calcium phosphate deposit as in the classical method.

The authors of the first paper found that fixation either in cold ethanol or cold formalin markedly depressed activity of the aromatic esters, but left the aliphatic ones substantially unaltered. They suggested that differences between the two methods in fixed paraffin

sections were therefore reduced or even extinguished. In the second paper Burgos and his co-authors re-emphasized these points and stressed, particularly, the differences in apparent localization which they obtained in kidney sections. Here, with glycerophosphate the precipitate was mainly in the striated borders of the proximal tubules, while with the naphthyl phosphates, and also with indoxyl phosphate, the deposit was predominantly cytoplasmic. Even more striking were the results obtained with a variety of inhibitors. Potassium cyanide (0·001 M), for instance, inhibited only glycerophosphatase activity, while 0·005 M-cysteine only affected the naphthyl phosphatase. The authors concluded that, in some tissues at least, separate enzymes are responsible for the hydrolysis of glycerophosphate and naphthyl esters. These conclusions have not been, but should be, tested by means of zymogram and other techniques.

Indoxyl Phosphate Methods

Very little work has been done on the localization of alkaline phosphatases using indoxyl substrates. The sodium salt of indoxyl phosphate was used by Seligman, Heymann and Barrnett (1954) and the result, in freeze-dried sections incubated at pH 9·5, differed to some extent from those obtained by alternative methods. Using this substrate, I found that localization was far from precise since the indigo was deposited in a macrocrystalline form. Far better results were obtained by Holt (1954), using the calcium salt of 5-bromoindoxyl phosphate in the presence of a ferro-ferricyanide oxidation catalyst as used in the indoxyl esterase method (Chapter XVI, p. 467). In this case the precipitate of 5,5′-dibromindigo was microcrystalline and the localization obtained was excellent.

The synthesis of halogen-substituted indoxyl phosphates is not easy and the more complex leucoindigo phosphates and thioindoxyl phosphates are not hydrolysed by tissue phosphatases. This important branch of phosphatase histochemistry remains undeveloped, therefore, at the present time.

Techniques for Specific Alkaline Phosphatases

Method for 5-nucleotidase (DW. 108)

In spite of what has been said earlier in this chapter it is possible, using the Gomori technique, to obtain useful results even at pH 7·5, provided that one is dealing with an active enzyme. This was shown by Pearse and Reis (1952), who used adenylic acid (adenosine-5-phosphate) as substrate in order to demonstrate the sites of activity of the specific 5-nucleotidase described earlier by Reis (1937, 1940,

1950). This enzyme has a pH optimum in the region of pH 7·8 in human tissues and pH 8·5 in the rabbit. Gomori (1949b) and Newman, Feigin, Wolf and Kabat (1950) estimated the presence of 5-nucleotidase by comparing two slides incubated at pH 9·2, one with adenylic acid, the other with β-glycerophosphate. The difference between the two was taken as corresponding to the activity of 5-nucleotidase, since adenylic acid is hydrolysed both by 5-nucleotidase and by alkaline phosphatase, whereas β-glycerophosphate is hydrolysed by non-specific alkaline phosphatase only. The result may be sufficiently clear when 5-nucleotidase is present in the examined tissue in the absence of alkaline phosphatase. If both enzymes are present, however, it is difficult to judge whether the heavier precipitates with adenylic acid are really due to 5-nucleotidase action.

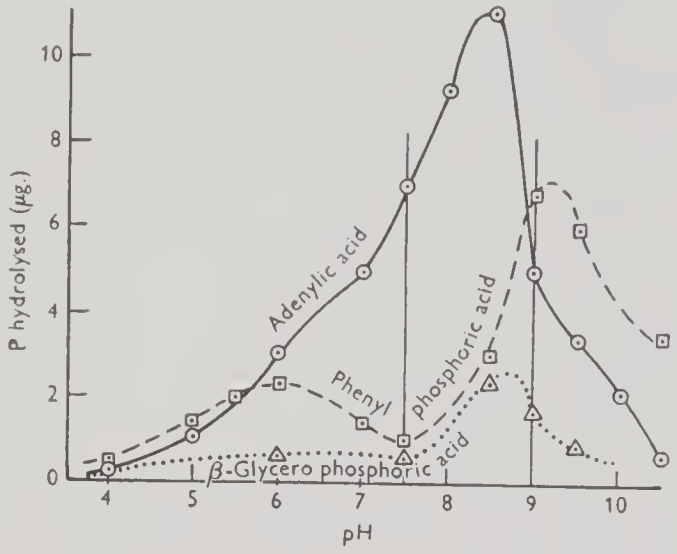


Fig. 112. Enzymic hydrolysis of phosphoric esters by an extract of rabbit lung. 5 mg. of tissue (dialysed extract), 20 min., temp. 38°, substrate concentration 0·0009 M. Details of technique as described by Reis (1951).

Little is known about the activation and inhibition of 5-nucleotidase, although the earlier studies of Reis (1937, 1950) had shown that magnesium activated this enzyme much less than it did non-specific alkaline phosphatase. Recent work by Ahmed and Reis (1958) has confirmed this and indicated that the most potent activator of 5-nucleotidase is Mn^{2+}, which produces a 60 per cent. increase in activity. Confirming earlier work by Kaye (1955), these authors showed that Zn^{2+} ions strongly inactivated the enzyme, but the strongest inhibition was produced by Ni^{2+}. As indicated in Fig. 113 below, a millimolar solution of a nickel salt almost totally inhibited 5-nucleotidase activity, while non-specific alkaline phosphatase activity was unaffected.

Application of these findings to the histochemical method of 5-nucleo-

tidase may be expected to increase its specificity to a considerable degree.

The different activity of alkaline phosphatase against various phosphoric esters has also to be taken into account. Fig. 112 shows the rate of hydrolysis of three phosphate esters *in vitro*, by slices of rabbit lung, as a function of the hydrogen ion concentration. At pH 9·0 it is obviously difficult to say whether the difference between adenylic acid and β-glycerophosphate hydrolysis is due to 5-nucleotidase activity or whether it is due to a different activity of alkaline phosphatase towards adenylic acid. The varying activity of alkaline phosphatase is illustrated by the hydrolysis of phenylphosphate which,

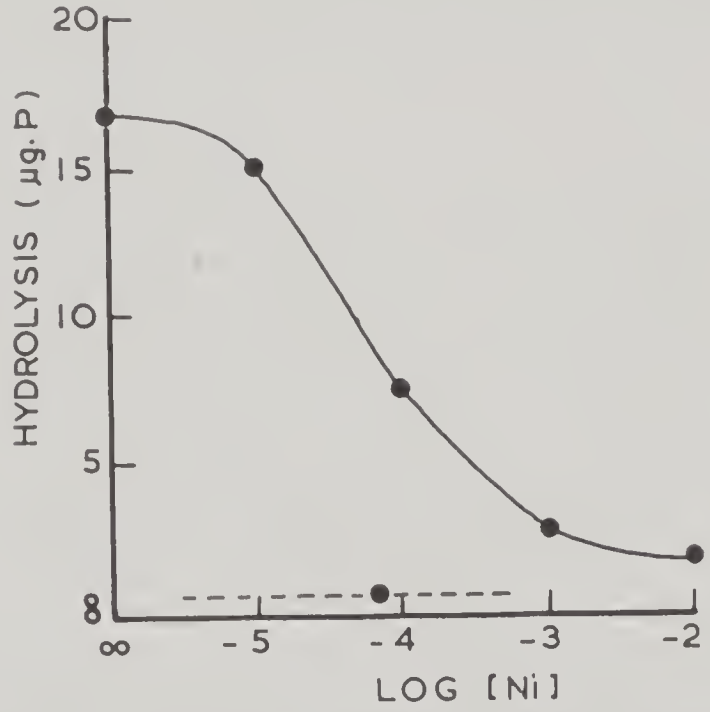


FIG. 113. Inhibition of aorta 5-nucleotidase by nickel. (20 mg. of tissue ; 30 min., pH 7·5, 38°.)

as was shown by King and Delory (1939), at pH 9·0 is about four times as fast as that of β-glycerophosphate, while that of adenylic acid is between the two. At pH 7·5, on the other hand, the rate of hydrolysis of phenylphosphate and β-glycerophosphate is very low, and the rate of hydrolysis of adenylic acid by non-specific phosphatase has a similarly low value at this pH. In subtracting such a small value from the total hydrolysis of adenylic acid a much smaller error is likely to be made than in subtracting the far larger values for non-specific hydrolysis obtained at pH 9·2. Incubation was therefore carried out by Pearse and Reis at pH 7·5, in spite of the known increase in solubility of calcium phosphate at this pH and the consequent increase in diffusion which was expected.

The disadvantage of using a low pH with the calcium nitrate method of phosphate precipitation is shown by a comparison of Figs. 114 and 115. Fig. 114 shows the disposition of alkaline phosphatase in the rat adrenal gland and indicates that it is fairly sharply localized and entirely in the cortex except for the walls of small arteries in the medulla. Fig. 115 shows the localization of 5-nucleotidase in the rat adrenal. All the staining observed in the photograph is due to this enzyme since a control section incubated with 0·005 M-phenyl phosphate at pH 7·5 was blank. This indicates that alkaline phosphatase, which can also hydrolyse adenylic acid at more appropriate pH levels, was inactive under the conditions of the experiment. The most intense staining in this case is in the medulla, but the presence of diffusion from this site is revealed by the staining of the nuclei in the cortex, which is most intense in a zone immediately surrounding the medulla. With incubation at pH 7·5, although in this case the production of calcium phosphate has been sufficiently rapid to cause local precipitation, localization is confined broadly to tissue regions and intracellular localization cannot be attempted. Figs. 116 and 117 show alkaline phosphatase and 5-nucleotidase, respectively, in serial sections of rabbit lung. No counterstain was used in either case.

Details of the method used by Pearse and Reis appear in Appendix 14, p. 874. By means of this technique only the sites of maximal 5-nucleotidase activity are demonstrated, even when cold formalin-fixed frozen sections are used, since for precipitation of calcium phosphate to occur at pH 7·5 production must be exceedingly rapid. The actual sites of lower 5-nucleotidase activity are not usually revealed, although diffuse staining of nuclei and other structures indicates the presence of 5-nucleotidase somewhere in the section. A positive histochemical reaction using adenylic acid at pH 7·5 cannot be considered as an artifact since it corresponds with chemical estimations and its intensity is, to a large extent, proportional to the time of incubation.

An essentially similar technique to that used by Pearse and Reis was employed by Antonini and Weber (1951) to demonstrate 5-nucleotidase in the medial coat of the aorta and other blood vessels. These authors used frozen sections after brief fixation in acetone. McManus *et al.* (1952) and McManus and Lupton (1953) used a Gomori type of procedure, with muscle adenylic acid as substrate, at a pH between 8·5 and 8·8. In the first paper a general study of human tissues was made, and the results agreed, on the whole, with those of Pearse and Reis. In the second paper a study of the enzyme in obsolescent renal glomeruli was reported. The general study may be criticized on account of (1) absence of alkaline phosphatase controls at pH 8·8 and 9·2, (2) the unduly long incubation times employed (for paraffin sections

15–24 hours), and (3) the excessively low concentrations of adenylic acid and Mg^{2+}. Later studies are those of Goebel and Puchtler (1954), who described the localization of the enzyme in renal infarcts, and of Otte (1958), who found a strong 5-nucleotidase in the surface layers of articular cartilage. Otte used a pH of 9·2 but observed no reaction with glycerophosphate as substrate except in the basal zone (of ossifying cartilage).

The calcium phosphate technique fails to give satisfactory results in fresh frozen or formalin-fixed frozen sections. An alternative technique using lead nitrate at pH 7·2 was employed by Naidoo and Pratt (1954) for use with freeze-dried sections. This gave a cleaner result, and Wachstein (1955) found this method gave a " very satisfactory and reproducible preparation " when applied to fresh frozen kidney sections. He also used cold formalin-fixed frozen sections, however, and suggested that with these the diffuse activity of the capillaries was suppressed, making the recognition of positive-staining tubules easier. Although this last effect seems undesirable, it is probable that the lead nitrate technique is the technique of choice for the demonstration of 5-nucleotidase in all types of tissue. Nevertheless, Novikoff (1958) maintains that variations in staining patterns (in rat liver) under varying conditions may be due to different 5-nucleotidases. It may therefore be justifiable, in many instances, to use a calcium and a lead method in parallel, on both fresh and formalin-fixed frozen sections.

Method for Aldolase (DW. 622)

This enzyme catalyses the splitting of a molecule of fructofuranose-1 : 6-diphosphate (hexose diphosphate) into one molecule each of 3-phosphoglyceraldehyde and α-phosphodihydroxyacetone. It is believed to play an important part in the reactions of glycolysis in yeast and in muscle. A small fraction of the total aldolase of rat liver is found in the nuclear fraction of homogenates. The remainder is present in the supernatant. Much higher specific activity occurs in skeletal muscle and somewhat less in cardiac muscle. Tung *et al.* (1954) investigated the substrate specificity of rabbit muscle aldolase and found that D-fructose-1,6-diphosphate and L-sorbose-1,6-diphosphate, which possess a *trans* configuration of hydroxyls on carbon atoms 3 and 4, were both hydrolysed more rapidly than tagatose-1,6-diphosphate which has the *cis* configuration. The affinity of crystalline muscle aldolase for fructose-1,6-diphosphate was much greater than that for fructose-1-phosphate, the respective Michaelis constants being 5×10^{-5} M and $3{\cdot}4 \times 10^{-3}$ M.

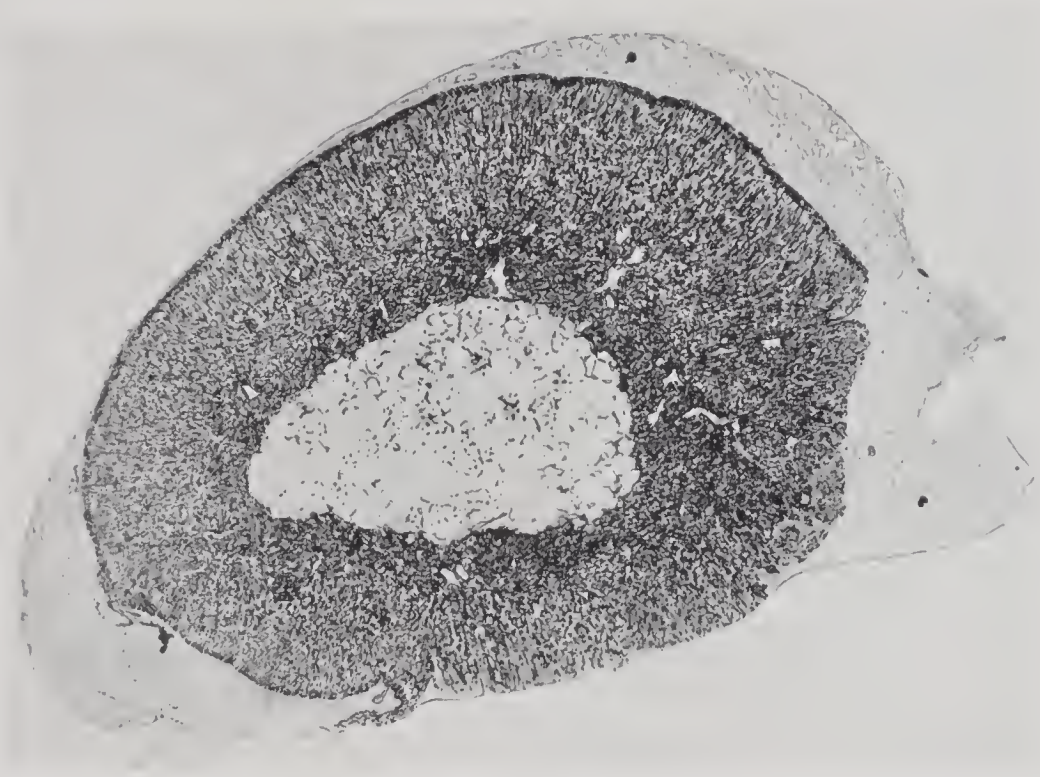


Fig. 114. Rat adrenal gland incubated for 3 hours with M/200 phenyl phosphate at pH 9·0. Disposition of alkaline phosphatase. × 19.

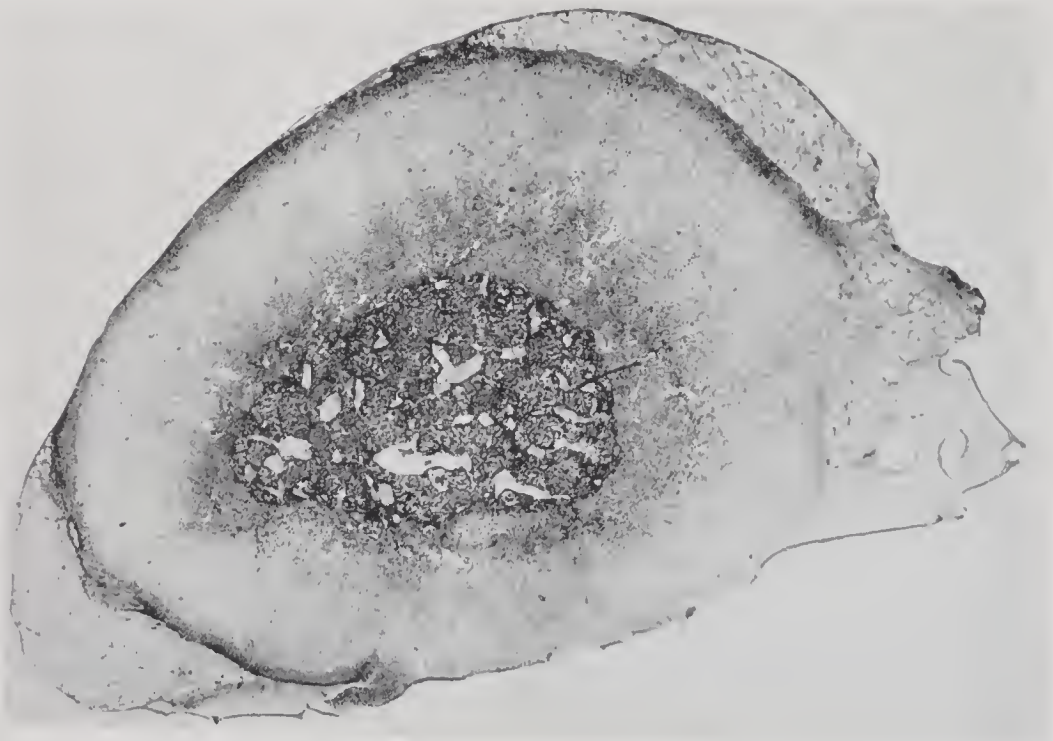


Fig. 115. Rat adrenal gland incubated for 3 hours with M/200 adenylic acid at pH 7·5. Disposition of 5-nucleotidase. × 19.

[*To face p.* 416.

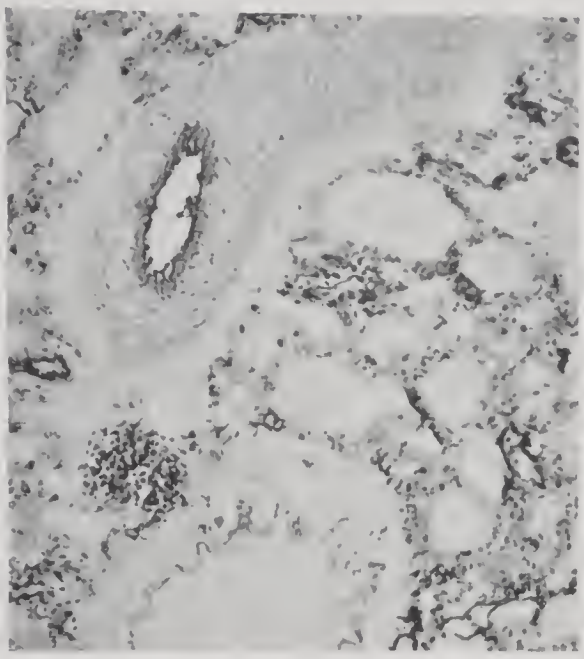


FIG. 116. Rabbit lung. Stained for alkaline phosphatase (phenyl phosphate, pH 9·0), which is present especially in the endothelium of a large artery. × 100.

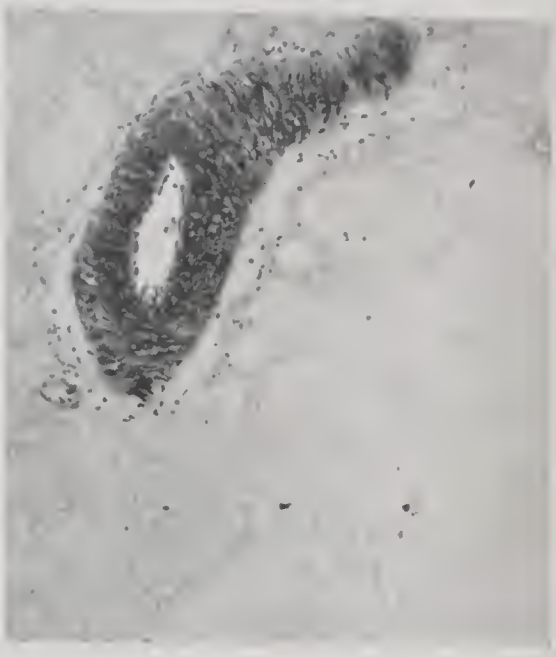


FIG. 117. Rabbit lung (serial section to Fig. 116). Stained for 5-nucleotidase (adenylic acid, pH 7·5), which is concentrated in the medial coat of the artery and absent from the endothelium. × 100.

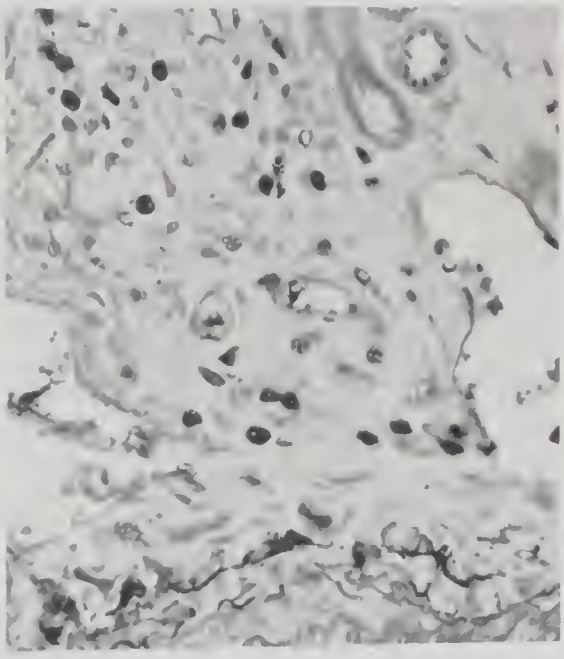
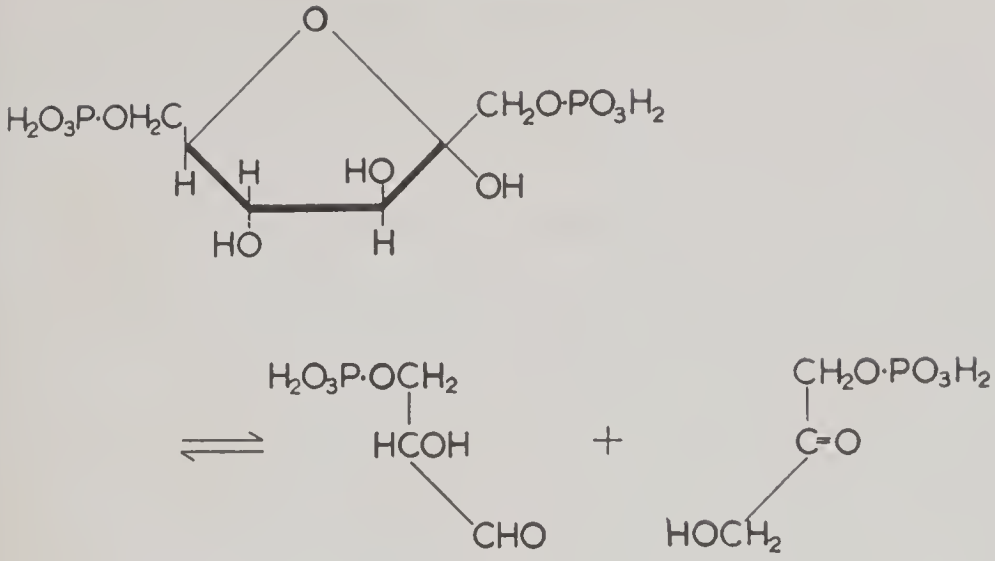


FIG. 118. Cold acetone-fixed, paraffin-embedded rat lung. Showing, *below*, alkaline phosphatase in adventitial fibres in the wall of an artery and, *above*, isolated lymphocyte and other nuclei also strongly stained. Sodium α-naphthyl phosphate—diazotized *o*-dianisidine. × 500.



Allen and Bourne (1943) used a Gomori type of procedure to demonstrate the phosphates produced by this reaction and thus to localize aldolase in the tissues. Further enzymic breakdown of the two triosephosphate reaction products was prevented by the presence of iodoacetate in the incubating medium and the reaction products were precipitated by a mixture of magnesium and ammonium chlorides in alkaline solution. Frozen sections of tissues fixed in 80 per cent. alcohol for 24 hours were used and these were incubated for 1–2 hours at 37°. The precipitated phosphates were revealed by their conversion to the cobalt salt and finally to the brownish-black sulphide.

I have no personal experience of this method, which appears on the surface to have several drawbacks. The authors stated that no confusion with alkaline phosphatase activity occurred on account of the different distribution of the two enzymes. Although in their earlier experiments fluoride was used to block alkaline phosphatase this procedure was later found unnecessary. It is difficult to see why alkaline phosphatase activity towards hexosediphosphate was lacking at the (alkaline) pH employed. The distribution of precipitate was found to be intracellular and nuclei and other structures were stained. This suggests that diffusion of the reaction products occurred and it is unlikely that localization was accurate, although the broad disposition of aldolase may well have been indicated. Few histochemical studies of aldolase seem to have been made, though Flexner *et al.* (1956) carried out studies on the tissues of the fœtal guinea-pig.

There would seem to be no *a priori* reason why an alternative technique should not be developed making use of the naphthoic hydrazide reagent to capture one or both of the two reaction products. This principle, suggested in the first edition of this book, has been successfully employed for demonstrating the tissue localization of the reaction product of the monoamine oxidase reaction (see Chapter

XVIII, p. 528). It would be necessary in this case, as in the monoamine oxidase reaction, to block pre-existing aldehydes or to compare the final production with a control section incubated in the absence of hexose diphosphate.

Methods for Adenosine Triphosphatase (DW. 180, 181)

This enzyme is responsible for the breakdown of ATP to ADP (adenosine diphosphate) with the release of free energy, a process utilized in muscle to supply the energy for contraction (see equation on p. 384). It has been suggested, moreover, that mitochondrial adenosine triphosphatase (ATPase) activity is in effect a reversal and diversion of the reactions responsible for the synthesis of ATP from ADP during oxidative phosphorylation (Lardy and Wellman, 1953). At least five enzymes are known which can dephosphorylate ATP, however. These are the specific calcium-activated ATPase of muscle, the magnesium-activated ATPase of muscle and other tissues, adenylpyrophosphatase, apyrase, and alkaline phosphatase. The position is further complicated by the fact that according to Myers and Slater (1957) rat-liver mitochondria contain four separate ATPases. The so-called adenylpyrophosphatase splits ATP into adenosine-5-phosphate and 2 moles of orthophosphate, and apyrase is a plant enzyme which transfers the terminal phosphate of ATP to some other compound. With these last two enzymes we are little concerned from the histochemical point of view. Most of our trouble arises from the activity of non-specific alkaline phosphatase. The possibility has to be considered that enzymes of the type which transfer adenylic acid might split ATP and leave inorganic pyrophosphate. Hydrolysis of the latter by an inorganic pyrophosphatase would release orthophosphate and give a positive histochemical reaction.

Using ATP as substrate, Glick and Fischer (1945, 1946) claimed that they could localize the specific adenosine triphosphatase of plant tissues, and they found the enzyme especially in the nucleoli of the cells of the wheat epicotyl.

The accuracy of their method depended on the absence of activity by non-specific alkaline phosphatase, which Moog and Steinbach (1946) showed could hydrolyse ATP. These authors criticized the method of Glick and Fischer and concluded that only non-specific alkaline phosphatases were demonstrated. They stated that localization of adenosine triphosphatase could only be made by assessing the difference between ATP-incubated and glycerophosphate-incubated slides. Glick (1946), however, maintained that the method would localize the specific enzyme whenever this was predominant. Maengwyn-Davies *et al.* (1952) demonstrated the activity of myosin ATPase in rat muscle, using fresh frozen sections and a Gomori type of procedure. They attempted

to control the specificity of the method by the use of a variety of activators and inhibitors and found that at pH 8·5 ATP was dephosphorylated by a specific SH-dependent enzyme. At pH 9·9, however, a different enzyme, possibly a pyrophosphatase, was concerned.

No further advances in the histochemistry of ATPase were made until Padykula and Herman (1955a and b) re-examined the whole question. These authors (1955a) compared the method of Maengwyn-Davies *et al.* (Appendix 14, p. 876) with a modified Gomori method of their own (p. 877), using fresh frozen sections in both cases. They found a much higher activity with their own method and considered that this was due mainly to the much lower concentration of salts in their medium. Another factor of importance was the substrate concentration (0·012 M compared with 0·005 M). In this case it was observed that the higher concentration of substrate produced nuclear staining and the lower concentration was therefore subsequently adopted.

In a second paper (1955b) Padykula and Herman tested the specificity of their method with a series of activators and inhibitors. They found that BAL (2,3-dimercapto-1-propanol), at about 5×10^{-3} M, would inhibit non-specific alkaline phosphatase activity while it enhanced the activity of ATPase. Sulphydryl inhibitors such as *p*-chloromercuribenzoic acid, on the other hand, abolished the activity of the specific enzyme and this inhibition was reversible with BAL or cysteine. In addition to these two types of phosphatase (ATPase, alkaline phosphatase) indicated by the above studies a third type of activity toward ATP was demonstrated. This was unaffected by the presence or absence of SH groups and was observed particularly in endothelia and in vascular smooth muscle. The application of the method of Padykula and Herman to fresh frozen tissues is shown in Figs. 119 and 120, p. 420. In thin (4 μ) sections incubated in protected media the intramitochondrial localization of the enzyme is clearly evident.

An alternative histochemical method for ATPase was designed and applied by Wachstein and Meisel (1957), using the Gomori lead nitrate medium at pH 7·2. This had previously been adapted successfully (Wachstein and Meisel, 1955) for the demonstration of 5-nucleotidase. With the lead nitrate method the bile canaliculi are particularly well shown, and Novikoff *et al.* (1958) were able to show that fragmented canaliculi in liver homogenates would react in the same way. In this paper the authors assessed the value of the Padykula-Herman and Wachstein-Meisel procedures by both biochemical and histochemical means.

It was apparent that with both methods enzyme activity was much

below its maximum. In homogenates incubated with the Padykula-Herman mixture only 20 per cent. ATPase activity was recorded, and with the Wachstein-Meisel medium only 15 per cent. Provided that loss of enzyme activity is not confined to the enzyme in any particular localization, leaving others unaffected, these percentages are of course perfectly acceptable in histochemical practice. Novikoff and his co-workers found that if formol-calcium fixation was superadded an additional loss of 80–90 per cent. occurred, and this is hardly to be tolerated.

The data obtained suggested that enzyme inhibition with the Wachstein-Meisel medium was caused by the high concentration of Pb^{2+} ions, a fact already established by Pratt (1954) in his biochemical and histochemical studies of the specific phosphatases in brain.

It was evident that the ATPase localized by both methods in the bile canaliculi was different from the one found histochemically and biochemically in the mitochondria. Already Novikoff *et al.* (1952) had shown that the ATPase of their nuclear fraction in their homogenates (now shown to be canalicular rather than nuclear) was activated by calcium. The mitochondrial enzyme is not activated by calcium, of course, and in fact it is almost totally inactivated in formol-calcium fixed sections. In Fig. 121 below is recorded the activity of rat-liver

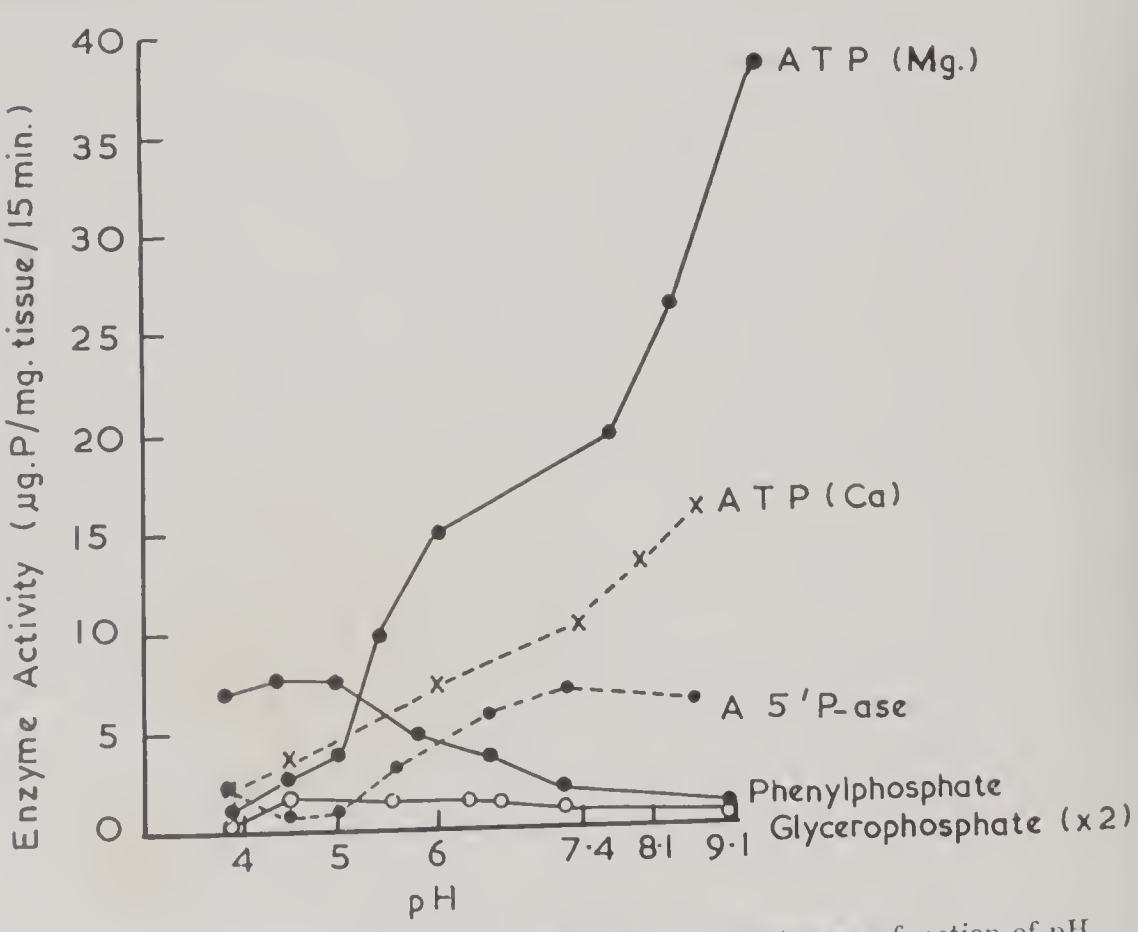


Fig. 121. Dephosphorylation by rat liver homogenates as a function of pH. (After Novikoff *et al.*)

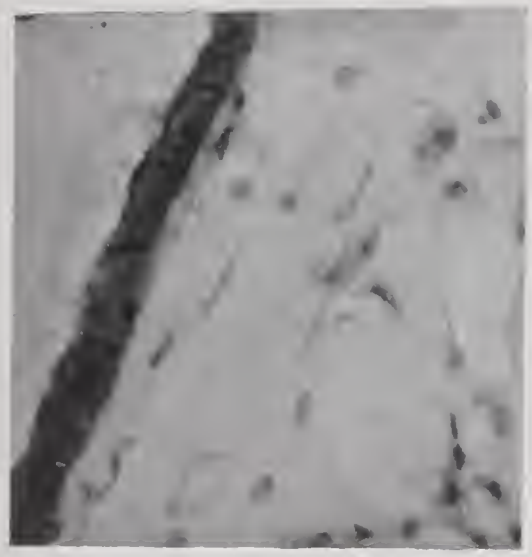


FIG. 119. Rat lung. Shows ATP-ase activity in the wall of a large blood vessel. Calcium method (Padykula-Herman). × 400.

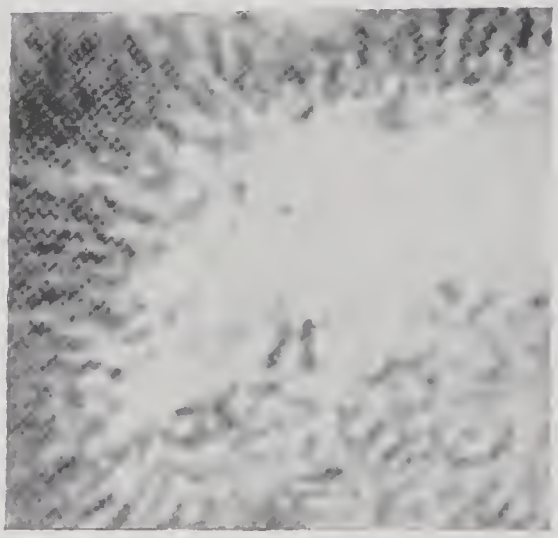


FIG. 120. Rat kidney. Osmotic protection with 7·5 per cent. PVP. Filamentous mitochondria showing ATP-ase. Calcium method (Padykula-Herman). × 2100.

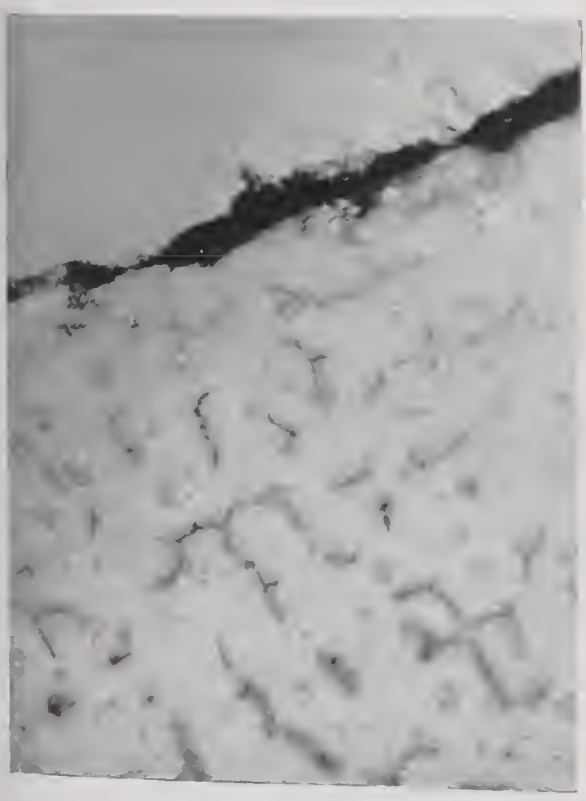


FIG. 122. Rat liver. ATP-ase outlining bile canaliculi and wall of portal vein. Lead method (Wachstein-Meisel). × 400.

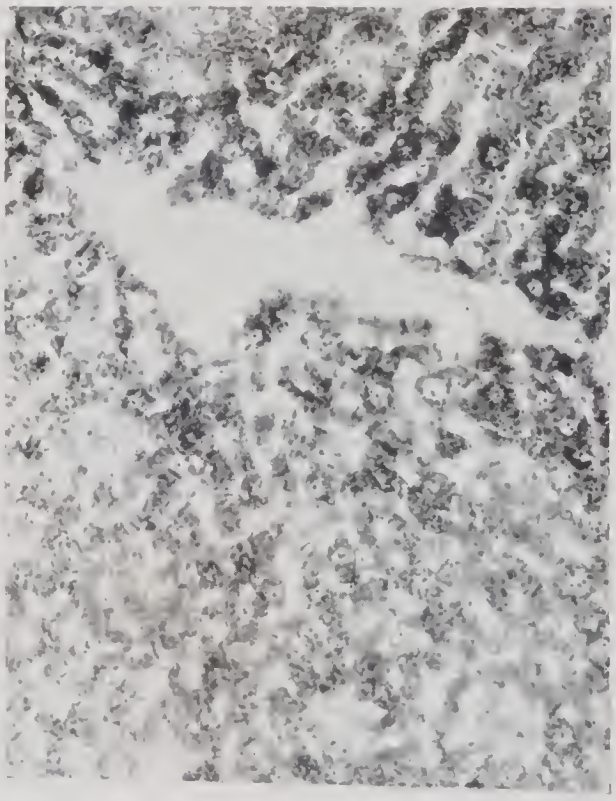


FIG. 126. Rat liver. Periportal distribution of glucose-6-phosphatase. Gomori-type method (Chiquoine). × 136.

[*To face p.* 420.

homogenates in the dephosphorylation of ATP, adenylic acid, phenyl phosphate and glycerophosphate.

At pH 7·2, the pH employed in the Wachstein-Meisel procedure, although the activity of both calcium-activated and magnesium-activated ATPase is only 50 per cent. of the maximum value, both values are nevertheless significantly higher than the activities of the homogenate towards the other substrates. In particular, the activity of non-specific alkaline phosphatase can obviously be ignored.

Novikoff *et al.* (1958) strongly recommend the Wachstein-Meisel procedure (Fig. 122, p. 420), and I agree with their conclusion, admitting that in the case of liver, at least, their experience is far greater than mine. Nevertheless, there may be instances where the mitochondrial enzyme is better shown by the calcium procedure of Padykula and Herman, especially in protected media.

Method for Thiamine Pyrophosphatase (possibly DW. 181)

The problem of the specific organic pyrophosphatases is far from being solved. The dephosphorylation of thiamine pyrophosphate was demonstrated in freeze-dried brain sections by Pratt (1954) using a lead nitrate type of procedure. This author found, as intimated above, that the concentration of Pb ions in the medium affected the amount of enzyme demonstrable histochemically. This effect is shown in Fig. 123, below.

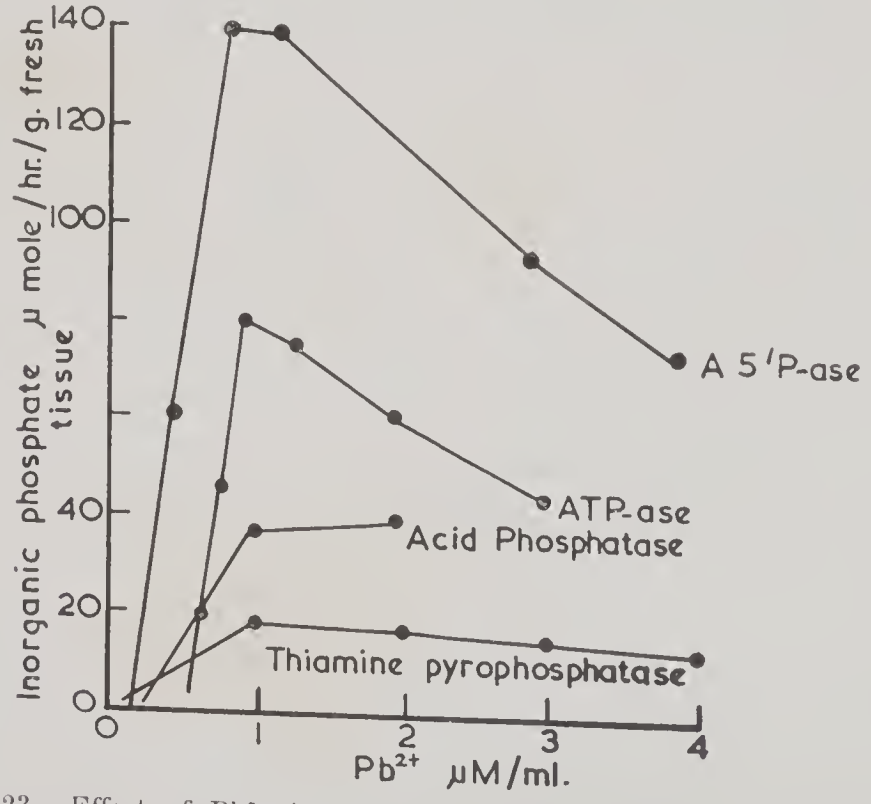


Fig. 123. Effect of Pb^{2+} ions on hydrolysis of thiamine pyrophosphate. (After Pratt, 1954.)

Pratt noted that at the lowest concentration of Pb^{2+}, where enzyme activity was at its highest, the sections stained unevenly. This was due to inefficient capture of liberated phosphate, and it serves to emphasize once again the double necessity in hydrolytic enzyme histochemistry to observe the principles set forth in Chapter XIII and to effect the best possible compromise within this framework.

A similar study of the histochemical dephosphorylation of thiamine pyrophosphate was carried out by Eränkö and Hasan (1954) on fresh frozen sections of rat kidney. At alkaline pH levels thiamine pyrophosphate and glycerophosphate gave identical pictures. In acid media, however, while the picture with glycerophosphate was that associated with non-specific acid phosphatase, the sections incubated with thiamine pyrophosphate showed strong activity, especially in the glomeruli. There was little or no activity in the convoluted tubules. The method used by Eränkö and Hasan is given in Appendix 14, p. 878.

Method for Inorganic Pyrophosphatase (DW. 177)

This enzyme has been described by Bailey and Webb (1944), and also by Mann (1944), Gordon (1950), Heppel and Hilmoe (1951) and McElroy *et al.* (1951), and its properties are therefore well recorded. Kurata and Maeda (1956) designed a method for its histochemical demonstration depending on the hydrolysis of a pyrophosphate-iron complex (Weden, 1930) and in fact they were able to demonstrate two enzymes, one active at pH 7·2 and the other at pH 3·7 to 4·0. Rogers and Reynolds (1949) have reported that pyrophosphate forms complexes of the type $Fe^{+++}(P_2O_7)^{-}$ with ferric salts, and Monk (1949a and b) and Jones and Monk (1950) have shown that these are relatively stable. This is a point of obvious importance if the localizations shown by Kurata and Maeda are to be considered accurate. Their curves for the activity of the alkaline enzyme are presented in Fig. 124, p. 423.

This shows the considerable activation produced by Mg^{2+} ions. Lohmann (1933) had already shown that inorganic pyrophosphatase of muscle and liver required Mg^{2+}, and later he extended his studies to yeast, red cells, brain, cereals, moulds and fireflies. Robbins *et al.* (1955) suggested that the need of the enzyme for Mg^{2+} is due to the fact that its true substrate is magnesium pyrophosphate. They found that pyrophosphate itself inhibited the activity of rat-brain pyrophosphatase.

In the presence of fluoride, however, Elliot (1957) has reported that the microsomal inorganic pyrophosphatase of guinea-pig liver is inhibited by magnesium rather than activated by it. Some discrepancy between these findings and those of Kurata and Maeda is obvious. The effect observed by Elliot is shown in Fig. 125, p. 423.

Marked stimulation of enzyme activity was also noted by Elliot

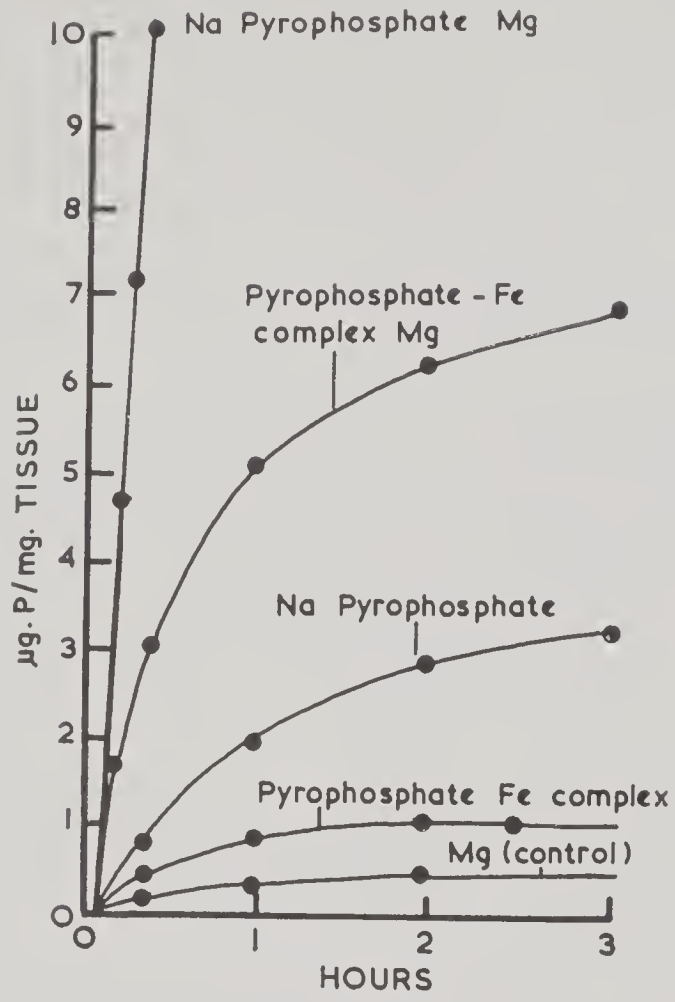


FIG. 124. Pyrophosphatase activity with various substrates at pH 7·6.

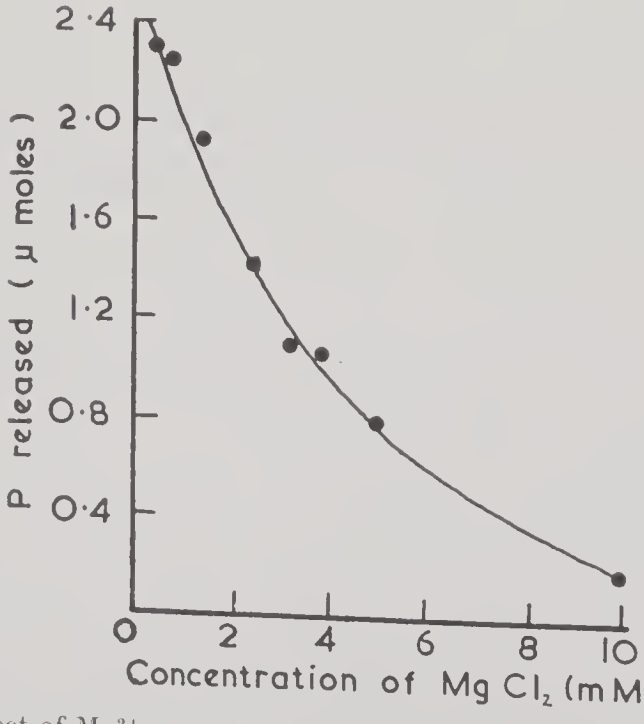


FIG. 125. Effect of Mg^{2+} on pyrophosphates in the presence of 0·5 M-fluoride.

after the addition of 0·005 M-ATP, the rate of hydrolysis being about double that found in control systems. Kunitz (1952) prepared a crystalline inorganic pyrophosphatase from bakers' yeast. This enzyme had no effect on organic esters such as ATP, ADP or thiamine pyrophosphate. It required Mg^{2+}, Co^{2+} or Mn^{2+} for activation and was antagonized by Ca^{2+}.

I have no experience of the method of Kurata and Maeda; details nevertheless appear in Appendix 14, p. 878.

Method for Inorganic Polymetaphosphatase (DW. 178)

This enzyme, whose biochemical characteristics have been described by Kitasato (1928a and b) and by Malmgren (1952), has a molecular weight of about 33,000. It has been found in a variety of genera, including moulds, yeasts and vertebrates, but its substrate, polymetaphosphate, is much more restricted in distribution. The enzyme breaks down its substrate, progressively, to the stage of pentametaphosphate.

A histochemical method for polymetaphosphatase was devised by Berg (1955), who tested homogenates of frog duodenum and frog embryos and found that they contained a magnesium-activated polyphosphatase with a pH optimum between pH 3·5 and 4·5. The enzyme was able to withstand a variety of fixatives and methods of preparation, the best being freeze-substitution (*iso*pentane-acetone, — 78°, 14 days). Preparation of the incubating medium is a somewhat tedious and critical procedure and, having no particular curiosity about this enzyme, I have not tested the method. Full details appear in Appendix 14.

Method for Glucose-6-phosphatase (DW. 113)

A specific enzyme capable of dephosphorylating glucose-6-phosphate has been found in a variety of mammalian tissues (Fantl and Rome, 1945 ; de Duve *et al.*, 1949 ; Swanson, 1950). It is absent from the liver in cases of glycogen storage disease (Cori, 1952–53) but present otherwise in all mammalian organs capable of releasing glucose into the blood stream (liver, kidney, small intestine, rectum). According to Shull *et al.* (1956) the level of glucose-6-phosphatase in the livers of hereditary obese hyperglycæmic mice is higher than in control animals. After cortisone, a marked increase in the enzyme level in homogenates has been noted by Weber *et al.* (1956). According to Beaufay and de Duve (1955) glucose-6-phosphatase acts on a variety of substrates, including phenyl phosphate, ethyl phosphate, α-glycerophosphate and fructose-6-phosphate. It is specifically inhibited by 1,5-sorbitan-6-phosphate (Crane, 1955). The enzyme is found predominantly, if not

exclusively, in the microsomal fraction of homogenates and it can therefore be used as a marker for the presence of microsomes.

The current histochemical method was introduced by Chiquoine (1953, 1955) using a Gomori type procedure with lead nitrate at pH 6·5–6·7. This pH optimum should, properly, cause it to be considered with the acid phosphatases in Chapter XV. It fits much better, however, into the sequence of enzymes discussed in this chapter. Chiquoine used fresh frozen sections in his studies, and these, or freeze-dried sections, are essential. Since both non-specific alkaline and acid phosphatase are capable of hydrolysing glucose-6-phosphate, it is obviously necessary to produce some proof of histochemical specificity. Chiquoine considered that, since formalin destroyed glucose-6-phosphatase but not acid or alkaline phosphatase, the absence of any reaction after 15 minutes' incubation of a formalin-fixed section with glucose-6-phosphate was sufficient proof of the specificity of the reaction. This is manifestly insufficient evidence, since the depressed activity of the non-specific phosphatases towards glucose-6-phosphate cannot be compared with their activity in fresh sections. The use of the specific inhibitor, sorbitan-6-phosphate, is perhaps indicated.

Details of Chiquoine's method are given in Appendix 14, p. 879, together with instructions for the conversion of the barium salt of glucose-6-phosphate to the potassium salt. Since the latter is now commercially available these may be redundant. Wachstein and Meisel (1956) have suggested a variation of the method, using the potassium salt as substrate, and this also is given in Appendix 14 and illustrated in Fig. 126, p. 420.

Fresh frozen sections, unless they are on the thick side (20 μ), tend to fragment during the later stages of both the above methods. Cold microtome sections mounted on coverslips are better, but the reaction is still sometimes patchy, especially when incubation is carried out with Chiquoine's original medium.

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CHAPTER XV

ACID PHOSPHATASES

Until recently histochemical methods for acid phosphatases have all been more or less unsatisfactory and the number of references in the literature to the use of such methods is still far smaller than the number of references to alkaline phosphatases. Histochemists and histologists have tended to overlook the importance of acid phosphatase in concentrating their efforts on the alkaline variety. With the advent of improved histochemical methods the position of acid phosphatase has been radically altered and there is now no reason why the different varieties of the enzyme should not be much more thoroughly studied.

The acid phosphatases are widely distributed in animal tissues, prostate, spleen and liver being three of the richest sources. There are a number of different acid phosphatases besides prostatic and red cell, the two main types with which the clinical pathologist is concerned. Prostatic acid phosphatase and its possible functions have been extensively studied by the Gutmans (Gutman and Gutman, 1938, 1940, 1941), and Herbert (1944, 1946) first showed that it could be differentiated from the normal serum acid phosphatase on account of its lability to alcohol. The distinctive red-cell acid phosphatase was described by King, Wood and Delory (1945), and Abu'l Fadl and King (1948a and b, 1949) found that it was totally inactivated by 0·5 per cent. formaldehyde, which did not affect the prostatic enzyme at all.

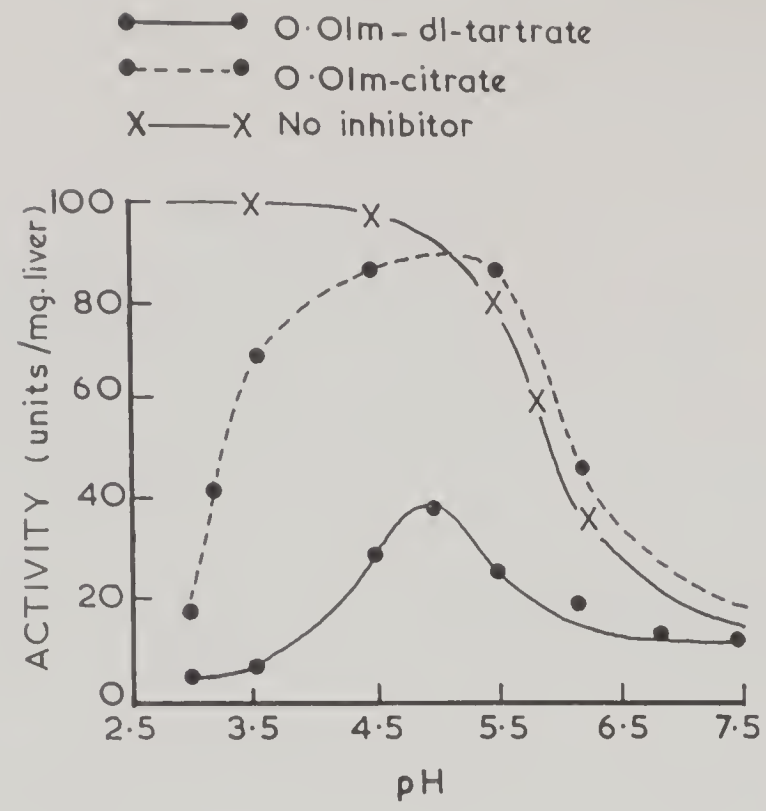
These observations have been the basis for most of the clinical biochemistry of the serum acid phosphatases, but histochemically their

Table 31

Percentage Inhibition of Acid Phosphatases at pH 5·0–5·5

Inhibitor	Liver *	Prostate †	Erythrocytes †	Adrenal cortex ‡
0·01 M-Mg^{2+}	Nil	Slight	Slight	Nil
0·0002 M-Cu^{2+}	— 85	Slight	— 95	— 86
0·01 M-cyanide	Nil	+ 12	+ 8	—
0·01 M-DL-tartrate	— 41	— 95	Nil	— 91
0·01 M-fluoride	— 53	— 96	— 8	—
0·5 per cent. H.CHO	— 68	Nil	— 100	— 30

* Goodlad and Mills (1957) . . 0·005 M-phenylphosphate, acetate buffer.
† Abu'l Fadl and King (1949) . 0·005 M-phenylphosphate, acetate buffer.
‡ Gordon (1952) . . . 0·01 M-β-glycerophosphate, acetate buffer.
Negative values indicate inhibition ; positive values activation.



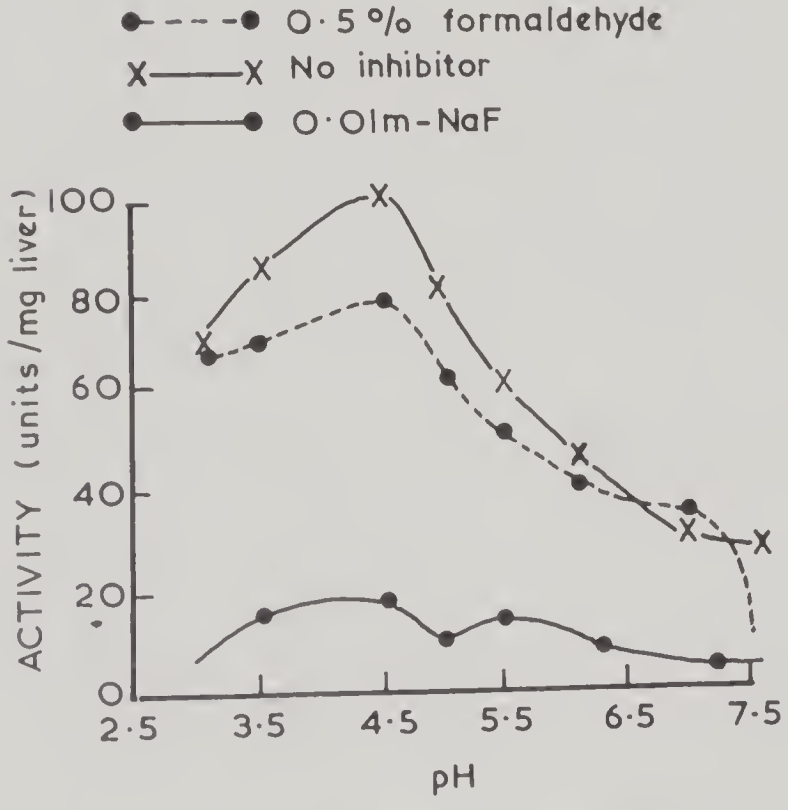


Fig. 127. Fig. 128.

Effect of Inhibitors on the pH-activity curve of acid phosphatase.
(After Goodlad and Mills.)

(0·005 M-phenylphosphate ; veronal-acetate buffer ; 38°.)

implications have not been adequately considered. The work of Folley and Kay (1936) indicated that there were three types of acid phosphatase which could be differentiated by their pH optima, their sensitivity to Mg^{2+} ions and their relative activity towards α- and β-glycerophosphates. Further studies by Roche (1950) definitely suggested that more than one acid phosphatase was present in the liver, and the recent work of Goodlad and Mills (1957) has produced further evidence that this is the case. Table 31, p. 431, which is derived mainly from the work of the last-mentioned authors, illustrates the effect of various inhibitors on acid phosphatase from four different sources.

It must be emphasized that species differences are apparent, since Abu'l Fadl and King found almost complete inhibition of human liver acid phosphatase by 0·01 M-tartrate between pH 4 and 6, whereas in the rat only 40 per cent. inhibition occurs.

It is clear from the findings of Goodlad and Mills that rat liver contains certainly two, and possibly three, acid phosphatases. One, having a pH optimum between 3·5 and 4·0, is activated by veronal and inhibited by citrate and tartrate as well as by fluoride. The other, having a pH optimum between 5·0 and 5·5, is inhibited by veronal and fluoride but not by citrate or tartrate. The third enzyme has a pH optimum of 6·0–7·0 with glycerophosphate as substrate. Figs. 127 and 128 show the effect of the various specific inhibitors on the pH activity curves of acid phosphatase in rat-liver homogenates.

It will be observed that the curve for fluoride inhibition, in the lower graph, shows evidence of two maxima, one at pH 4·0 and the other at pH 5·5. Observations on the inhibition of particularly the prostatic acid phosphatase by α-hydroxy carboxylic acids have been made by Anagnostopoulos (1953) and by Kilsheimer and Axelrod (1957).

Techniques for the Non-specific Acid Phosphomonoesterases (DW. 107)

Methods Depending on Deposition of Lead Phosphate

The original technique for demonstrating acid phosphatases was introduced by Gomori (1941). It depended, as did his alkaline phosphatase method, on the liberation of phosphate ions from organic phosphate esters. The reaction is buffered at pH 4·7 to 5·0 and it is therefore impossible to use calcium ions to form a precipitate of calcium phosphate, since this product is completely soluble in the acid range. Lead nitrate is used instead of calcium nitrate in the incubating solution and its phosphate is insoluble at pH 5·0. The precipitated lead phosphate is converted by means of yellow ammonium sulphide into the brown sulphide of lead.

Wolf, Kabat and Newman (1943), in the course of a study on the

distribution of acid phosphatases in which they used the Gomori technique with a variety of phosphate esters, noted that non-specific but localized deposits of lead salts occasionally occurred. These superficially resembled the accepted histochemical distribution of the enzyme, especially in spleen, testis and brain. Moog (1943) reported a similar capriciousness of the Gomori technique when applied to the spinal cords of chick embryos. Hard and Lassek (1946) and Lassek (1947) also criticized the technique. The latter described the persistence of staining in the axons of the brain stem and spinal cord of the cat, monkey and man, and in the sciatic nerve of the cat, after manœuvres which certainly destroyed all enzymes. He suggested that the appearances produced by the Gomori technique were artifacts due to non-specific deposition of lead.

The more recent studies of Newman, Kabat and Wolf (1950) throw some light on the subject. These authors re-examined the specificity of the method in view of the implication that methods using lead salts for the histochemical localization of phosphatases and other esterases demonstrated nothing other than the affinity of various tissue components for lead salts in the acid range. They compared the effect, at various pH levels, of incubation with buffered lead salts only, and of incubation with lead-glycerophosphate mixtures. Their results showed that non-specific staining by lead varied directly with the pH, occurring infrequently at pH 5 and below, increasing in intensity and regularity above pH 5·3 and decreasing again in intensity only between pH 6·0 and 6·8. At pH 5·6 to 6·8, the distribution pattern in some organs, notably kidney, intestine, testis, epididymis, heart and brain, resembled that described for alkaline phosphatase. In most tissues the lead effect was noted especially in the nuclei, in cuticular borders and at surface interfaces. It was not influenced by 0·01 M-sodium fluoride, which has been shown chemically and histochemically to inactivate acid phosphatases, so that any staining in the absence of substrate, or the persistence of staining in the presence of substrate and 0·01 M-sodium fluoride, was due solely to lead. The lead effect was also noted to be unaffected by formalin or by 95 per cent. alcohol, both of which were considered to inhibit acid phosphatases. Both the non-specific staining by lead and the true enzymic staining were prevented by heating the sections at 80° in distilled water for 10 minutes or by treatment with 5 per cent. trichloracetic acid for 10 minutes. Both these manœuvres might be expected to depolymerize nucleic acids in addition to destroying any enzyme present, thus reducing the number of acid groups available for combination with the metal.

More recent information concerning the mechanism by which lead becomes attached to tissue components has come from the work of Clarkson and Kench (1958). These authors have shown that when

5 μg. of lead (as $PbCl_2$) is added to 5 ml. of whole blood, over 95 per cent. is rapidly attached to the red cells. Because of the failure of a variety of potential competitors to interfere with this reaction Clarkson and Kench considered that the lead must be operating either as a metal complex or as a chelate compound or, perhaps, as a dispersed colloidal metal salt.

Millet and Jowett (1929) showed that, in solutions having the same ionic strength and pH as plasma, lead orthophosphate was precipitated when the concentration of lead rose to about 0·0001 M. The concentration in the standard acid phosphatase medium is about 0·003 M. When, however, organic substances are present, as they are in plasma, a process of "peptization" (Lovelock, 1954) takes place and the lead phosphate sol is prevented from coagulating and forming a precipitate. Clarkson and Kench have stated that metal-salt sols, except at their equivalence point, carry electrical charges and can be removed from suspension by non-specific absorbents. This process presumably occurs under histochemical conditions of incubation. A strong pH effect was noted. Uptake of lead by cells was markedly reduced when the pH was lowered from 6·6 to 6·0, in which range, according to the authors, the highly insoluble lead orthophosphate is transformed into the more soluble lead monohydrogen phosphate. This point is of obvious histochemical significance.

A further important point concerns the charge on the lead phosphate sol. If excess phosphate is present the sol bears a negative charge, but if the concentration of free phosphate ions is low, as it is under histochemical conditions, the net charge becomes positive. Thus Clarkson and Kench found that combination of lead sol with the negatively charged erythrocyte occurred more rapidly in saline than in plasma or other phosphate-containing solutions. On the above evidence one would expect histochemical non-specific absorption of lead to be confined to structures carrying a negative charge, and this is certainly observed in practice.

Newman *et al.* concluded that valuable information might still be derived from the Gomori procedure, carried out at pH 4·7 with glycerophosphate as substrate, provided that two control sections were used. One control should be incubated with substrate to which 0·01 M-sodium fluoride has been added and the other in the buffer + lead nitrate only. The non-specific effects noted in these two sections are subtracted from the total picture to give the result due to enzymic action alone.

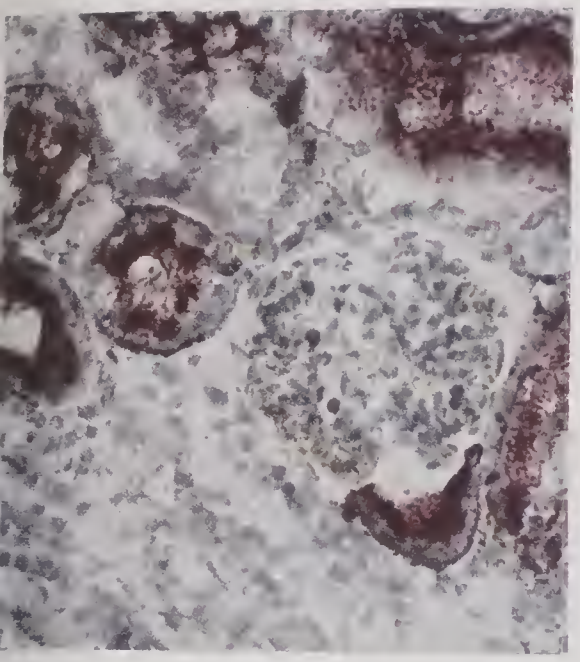
Gomori's Improved Technique. Gomori (1950), after examining the causes of failure of his original method, introduced an improved technique. Discussing the various factors which were responsible for such failures, he admitted that patchy fixation might account for certain differences in intensity between one part of the section and another,

but agreed with Sulkin and Kuntz (1947) that this factor could not explain all the observed phenomena. Particularly difficult to explain on this basis were the failures with some but not all sections cut from the same block, and failures with a fresh batch of incubating fluid prepared in the same way as a previously successful batch. In his experimental investigations Gomori used serial sections of the same block of tissues and incubated these with glycerophosphate at pH 5·0, altering variously the concentrations of substrate, buffer and lead salt. The original substrate concentration of 0·005 M was found to be inadequate, but uniformly good results were given with substrate concentrations between 0·014 and 0·05 M (buffer 0·08 M, lead nitrate 0·037 M in the final solution). On varying the buffer concentration Gomori found that this should not exceed that of the substrate by more than a factor of 6. A ratio of 5 or 6 to 1 was found to be optimal in the case of glycerophosphate. Increasing the concentration of lead increased the intensity and uniformity of the reaction only within narrow limits.

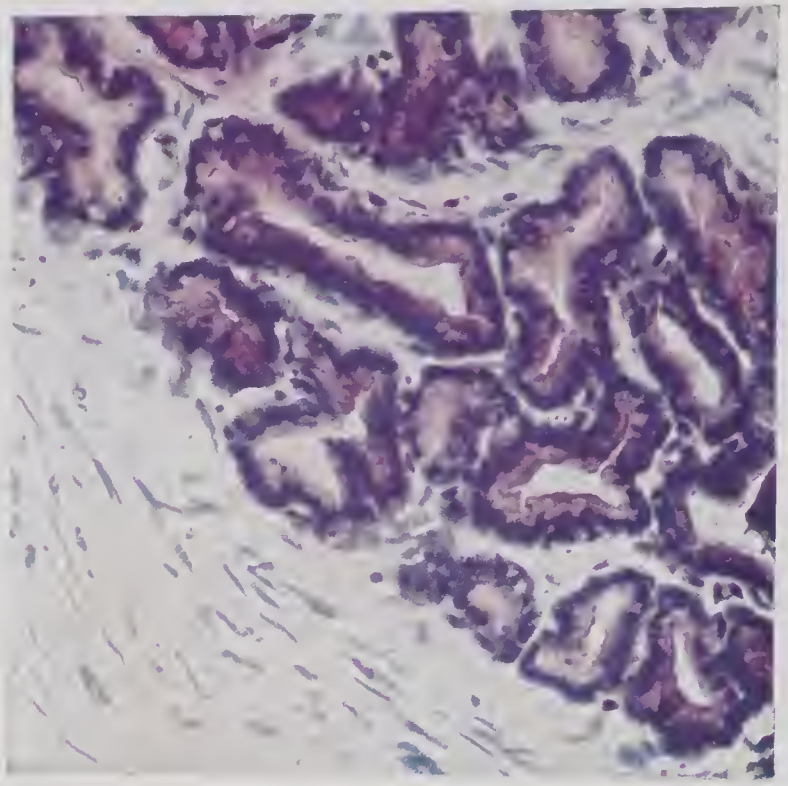
Gutman and Gutman (1938) showed that in the case of acid phosphatase the rate of hydrolysis was increased with rising substrate concentration up to 0·1 M, and Gomori emphasized the need to keep the substrate concentration high, to use substrates which were hydrolysed rapidly and to minimize the solubility of the reaction product as far as possible. Since raising the concentration of lead depresses the concentration of substrate, a low concentration of lead was employed. Similarly, high concentrations of buffer increase the solubility of the reaction product and one should therefore use the minimal amount of buffer necessary to stabilize the pH. The final concentrations arrived at by Gomori are given in the description of his improved method in Appendix 15, p. 881.

Gomori also attempted to increase the efficiency of his method by employing substrates which were hydrolysed faster than glycerophosphate. Phenylphosphate could not be used since its lead salt is completely insoluble, but the monophosphates of catechol and resorcinol were successfully employed as histochemical substrates. These offered no advantages over glycerophosphate, however, and their routine use was not advised. In spite of the observations of Moog (1943) that intensification of the acid phosphatase reaction could be obtained by means of manganese ions (0·01 M-manganese sulphate) or ascorbic acid (0·01 M), Gomori did not recommend the use of activators.

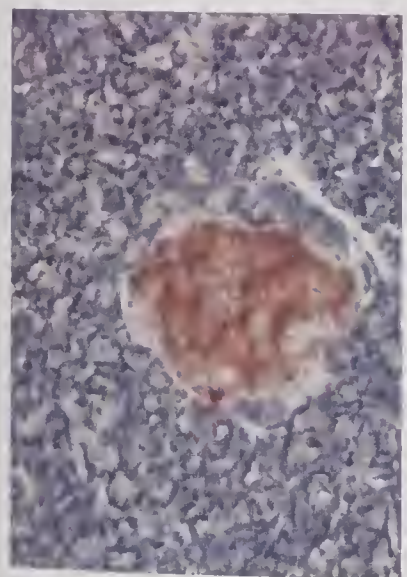
Other Modifications. Goetsch and Reynolds (1951) stated that two major factors radically affected the results observed when using Gomori's (1941) technique, at pH 4·3, on acetone-fixed paraffin sections. These were (1) the technique of mounting the section on the



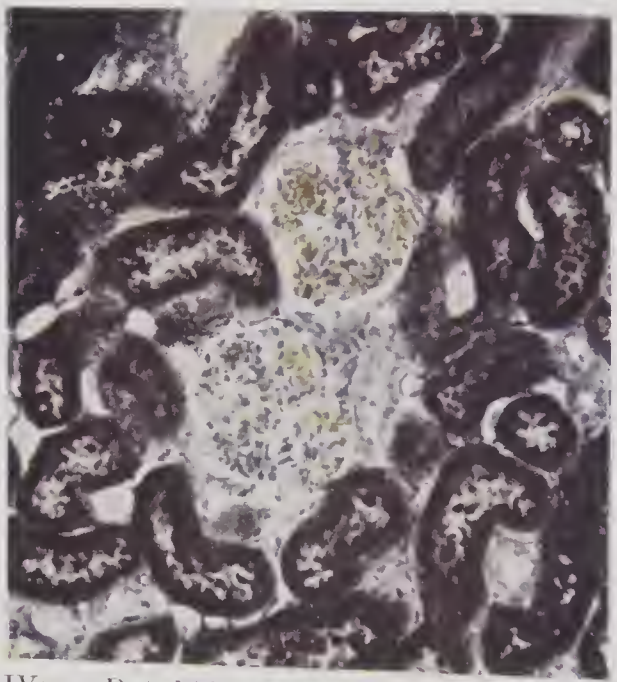
IVA. Guinea-pig kidney (10 μ frozen section). Alkaline phosphatase in tubules and in a polymorph. Incubation 15 minutes. Coupling azo dye method, Mayer's hæmalum. × 220.



IVB. Dog prostate (10 μ frozen section). Acid phosphatase in the cytoplasm of the acinar cells. Incubation half a minute. Coupling azo dye method, Mayer's hæmalum. × 235.



IVC. Rat pancreas. Islet of Langerhans. Acid phosphatase in the beta cells, no staining of the peripherally situated alpha cells. α-Naphthyl phosphate method. × 60.



IVD. Rat kidney (10 μ frozen section). Non-specific esterase in the convoluted tubules (cf. Plate IIIB). α-naphthol acetate method. × 130.

[*To face p.* 436.

slide, and (2) the method of deparaffinization and hydration. They recommended that the temperature of the water bath used for flattening the sections should be no higher than 38°–40°, or just sufficient to melt the paraffin. Higher temperatures caused inactivation, beginning at the edges of the slide. In this context it is interesting to note that Woodard (1951) showed that at pH 8·0 or higher 1 hour's exposure to 37° was sufficient totally to inactivate prostatic acid phosphatase. Marked inactivation of the enzyme in the sections was caused by the use of egg albumin as an adhesive and it was recommended that no adhesive be used. Three different procedures were used in preparing the slides for incubation: (1) the slides were brought to water via toluene and alcohol ; (2) the slides were brought to water via toluene, toluene-acetone and acetone, respectively ; (3) the slides were not deparaffinized at all. After incubation, slides treated by procedure (1) showed the least amount of precipitate, those treated by procedure (2) showed slightly more, and those treated by procedure (3) showed very much more precipitate. Eränko (1951b and c), observing that the results he obtained with the Gomori (1941) technique on acetone-fixed paraffin sections were entirely inadequate, used the same technique on frozen sections, cut preferably from fresh material or, failing this, from material fixed in 4 per cent. neutral formalin for 2–6 hours at room temperature or for 12–24 hours at 0°–3°. The results he obtained with these modifications were consistently reproducible and no patchy negative areas were seen.

An interesting suggestion was made by Tandler (1953), who replaced the lead ions of the usual media by 0·04 M-Co^{2+}. Unfortunately, this modification could not be employed at pH 5·0 since at this pH the precipitation of phosphate was incomplete. At pH 6·0 to 6·5, however, acetone-fixed tissues produced in 12 hours a deep blue colour at the sites of enzyme activity. This was converted into black CoS in the usual way. It is probable that investigations into the properties of other metallic ions having phosphates insoluble between pH 3·0 and pH 7·0 would produce alternative phosphate precipitates for use in the histochemistry of acid phosphatase.

Another modification, which has attracted less attention than it deserves, is that of Takeuchi and Tanoue (1951). This employs a somewhat higher pH than usual (up to pH 6·0 may be employed), and final development of the lead phosphate precipitate is achieved with an ammoniacal silver nitrate solution. Details appear in Appendix 15, p. 881.

Practical Considerations of the Method. In order to decide which, if any, of the reported modifications of the Gomori technique might be recommended for general use, Grogg and Pearse (1952), in a series of experiments, compared Gomori's 1941 with his 1950 technique,

using (1) acetone-fixed paraffin sections, (2) cold formalin-fixed frozen sections, and (3) freeze-dried sections, and both Gomori techniques with the modification of Goetsch and Reynolds using paraffin sections only. In all cases control sections were incubated (*a*) without substrate, and (*b*) with substrate plus 0·01 M-sodium fluoride. Using acetone-fixed sections of rat liver, it was found, in agreement with Gomori (1950), that the ratio of buffer to substrate was the most important factor in obtaining uniform results. With a substrate concentration of 0·005 M either the sections were negative or precipitation of lead phosphate was patchy. With a concentration of 0·01 M uniform pictures were obtained and the amount of precipitate, over a short period, appeared to be proportional to the incubation time. Sections treated according to the recommendations of Goetsch and Reynolds, and incubated without removal of paraffin, always gave patchy results. With formalin-fixed frozen sections of rat liver the 0·005 M substrate failed to give uniform results, and the precipitate appeared first in the nuclear membrane and nucleoli ($\frac{1}{2}$–2-hour incubation). After 4 hours' incubation the nuclei were completely black and a few fine cytoplasmic precipitates were visible, along the bile canaliculi. After prolonged incubation (16 hours) varying pictures were obtained. One section (Fig. 129, p. 440) showed coarse precipitation in the nuclei, but another identically-treated section (Fig. 130) showed staining of the cytoplasm only, the nuclei remaining colourless. Using the 0·01 M substrate concentration a completely different precipitation pattern was obtained. After short incubation, precipitates occurred along the bile canaliculi and the nuclei were scarcely stained except in the Kupfer cells. After longer incubation (1–4 hours) the precipitates in the cytoplasm became coarser and the nuclei more definitely stained (Fig. 131). Storage of sections did not alter the distribution pattern of precipitate, but with prolonged formalin fixation (208 instead of the usual 16 hours) practically only the nuclei were stained. An essentially similar result to the last was obtained by using the Gomori (1941) medium on frozen sections fixed for 16–40 hours only (Fig. 132).

Using the Gomori (1950) technique on freeze-dried sections of rat liver a precipitation pattern closely resembling that seen in frozen sections was observed, but it was markedly less uniform. In some parts the precipitates were in the nuclei and in others in the cytoplasm along the bile capillaries and in the Kupfer cells.

It was concluded from the above experimental work that if the lead-phosphate technique was to be used at all for the histochemical demonstration of acid phosphatases, the best procedure was to employ Gomori's (1950) method on 16-hour cold formalin-fixed frozen sections, with an incubation of $\frac{1}{2}$–4 hours. This modification of the method is given in Appendix 15, p. 881. It must be emphasized, however, that

altering the concentration of substrate alters the intracellular precipitation pattern and so, to a lesser extent, does variation in the time of formalin fixation. In spite of this, acetone fixation and paraffin embedding, with its 95 per cent. destruction of the enzyme, is quite unacceptable as an alternative. Since the distribution of precipitate has thus been shown to be variable, depending on physicochemical conditions rather than on enzyme distribution, it is necessary to conclude that the lead phosphate technique, at best, gives only an approximate or topographical picture of the distribution of acid phosphatase in the tissues.

Distribution of Acid Phosphatases by the Gomori Method. The classical papers dealing with the distribution of acid phosphatases in the tissues are those of Gomori (1941) and of Wolf, Kabat and Newman (1943). There are a few other papers dealing primarily with the application of the technique ; with those that have already been quoted the following list is representative, if not comprehensive. *Brain and Neurones:* Bodian and Mellors (1944), Smith (1948). Josephy (1949), Eränko (1951a), Naidoo and Pratt (1951), Mottet and Barron (1953), Walter (1954–55). *Peripheral nerves:* Bartelmez and Bensley (1947). *Blood:* Rabinowitch (1949a and b), Woodard (1950), Weiss and Fawcett (1953). *Nuclear structures:* Wachstein (1945), Rabinowitch (1949c). *Adrenal cortex and medulla:* Soulairac, Desclaux and Tesseyre (1949a and b), Eränkö (1951b), Hillarp and Falck (1956). *Fibroblasts:* Noback and Paff (1951). *Prostate (Benign and Malignant):* Downey *et al.* (1954), Brandes and Bourne (1954), Mathes and Norman (1956). It is notable that in a biochemical study of prostatic acid phosphatases Schwartz *et al.* (1953) showed that in the normal gland two peaks (pH 4·0 and 5·5) were present, while in the malignant gland only one peak (pH 5·5) could be found. *Skin:* Moretti and Mescon (1956), Spier and Martin (1956). *Metanephros:* Eränkö and Lehto (1954).

Simultaneous Coupling Azo Dye Methods

The Seligman-Manheimer Technique. The principle of the Menten-Junge-Green coupling azo dye technique for the demonstration of alkaline phosphatase was adapted by Seligman and Manheimer (1949) to the histochemical demonstration of acid phosphatase. These authors found that α-naphthyl phosphate was hydrolysed more rapidly than the β-naphthyl ester at acid pH levels and this substrate was therefore employed, in the form of its calcium salt. Since it was found that alcohol had a greater effect than acetone in inactivating acid phosphatase, acetone-fixed paraffin-embedded sections were carried through xylene and acetone to water and incubated in 0·3 M-acetate buffer at pH 5·0 in the presence of substrate (approximately

0·0018 M), 4 M-NaCl, and anthraquinone-1-diazonium chloride. Although some text-books suggest the contrary, phenols can couple efficiently with diazonium salts in acid solution. With a number of diazonium salts, nevertheless, coupling with α-naphthol will not take place at pH 5·0, and if it does occur the resulting dye often differs from the one which is formed at an alkaline pH. After a search for a suitable diazonium salt Seligman and Manheimer selected anthraquinone-1-diazonium chloride, which they considered to be very stable in aqueous solutions, making possible the necessary prolonged incubations without renewing the medium. They also found that a temperature of 20° could conveniently be employed. The reaction, with calcium α-naphthyl phosphate and anthraquinone-1-diazonium chloride, follows the pattern of the coupling azo dye method for alkaline phosphatase, the equation for which is given on p. 402.

In order to increase the low solubility of the substrate in the aqueous medium Seligman and Manheimer added a small amount of Aerosol (an alkyl sulphonate detergent). This practice cannot be recommended since detergents of this type markedly increase the solubility of the azo dye reaction products so that widespread diffusion occurs. The high concentration of sodium chloride was used with the idea of decreasing the solubility of acid phosphatase in the incubating medium (acetone-fixed acid phosphatase is still appreciably soluble in water). In my view, however, some of the efficiency of sodium chloride in producing clear pictures free from diffusion is due to salting out of the azo dye reaction product.

Seligman and Manheimer found that for human and dog prostate the incubation period was as short as 1 hour, but for all other tissues it was in the region of 12–24 hours. With these longer periods they observed that the nuclei were invariably more densely stained than any other tissue components, but with the shorter incubation period I have observed that this staining does not always occur. Fig. 133 shows the result of applying the Seligman-Manheimer technique to human prostate and incubating for 45 minutes. No staining appears in the nuclei as a whole and the reaction is mainly confined to the contents of the prostatic acini. The connective tissues around areas of high activity, however, and some of the nuclei therein, are appreciably coloured. This is most likely to be due to diffusion of enzyme before and during fixation. The degree of non-specific background staining is negligible with the short incubation time but, as decomposition of the diazotate proceeds, the background structures are stained progressively yellow and finally brown. In their method Seligman and Manheimer terminated the incubation by washing in water, and they mounted their sections in Kaiser's medium, since the brownish-red pigment was soluble in organic solvents. Control sections were incubated in the

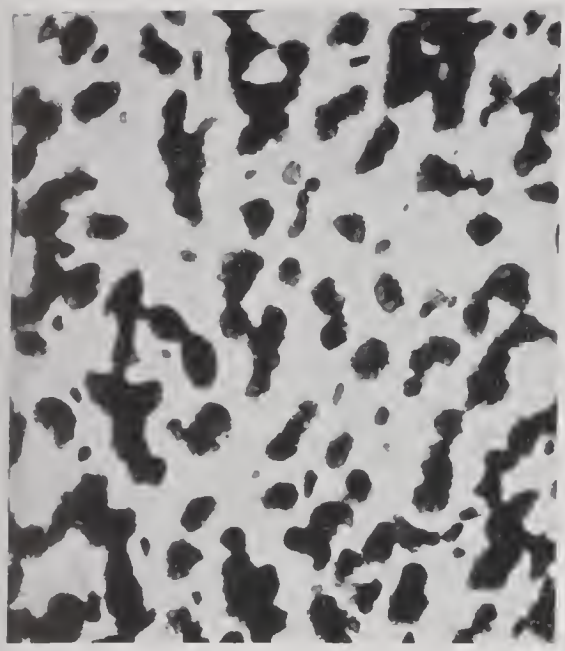


FIG. 129. Frozen section (10 μ) of rat liver after 16 hours' cold formalin fixation and 16 hours' incubation in Gomori's (1941) medium for acid phosphatase. The precipitate is chiefly present in the nuclei. × 220.

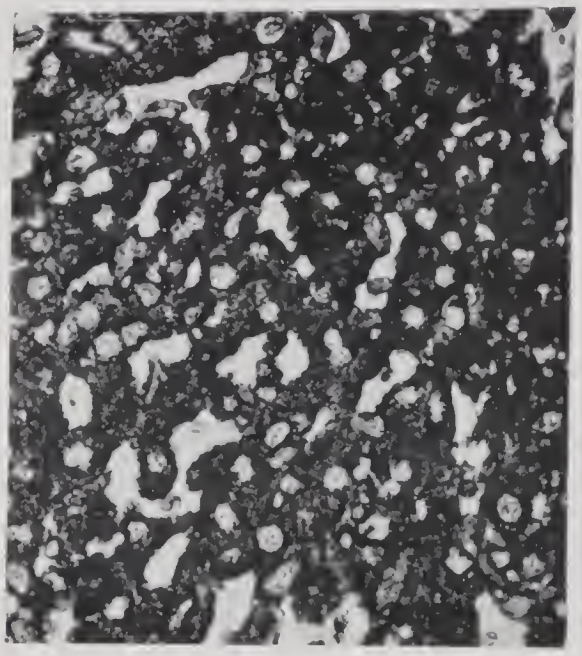


FIG. 130. As Fig. 129. Section similarly treated. The precipitate is largely absent from the nuclei. × 220.

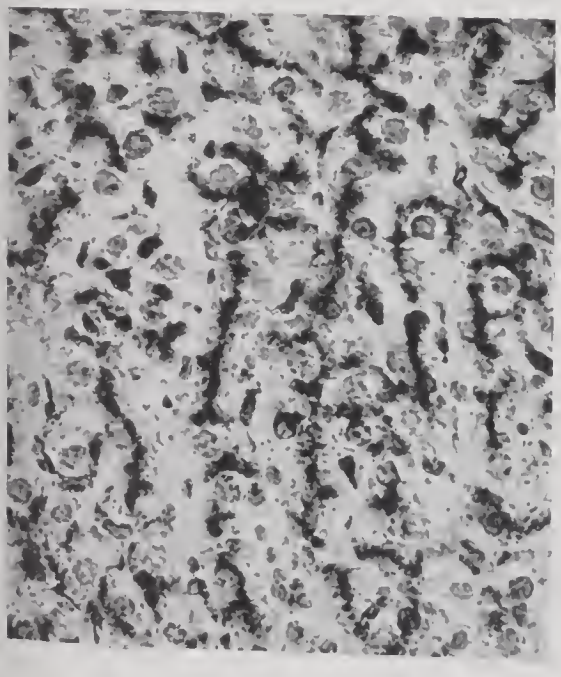


FIG. 131. Frozen section (10 μ) of rat liver after 16 hours' cold formalin fixation and 1 hour's incubation in Gomori's (1950) medium. Eosin. × 220.

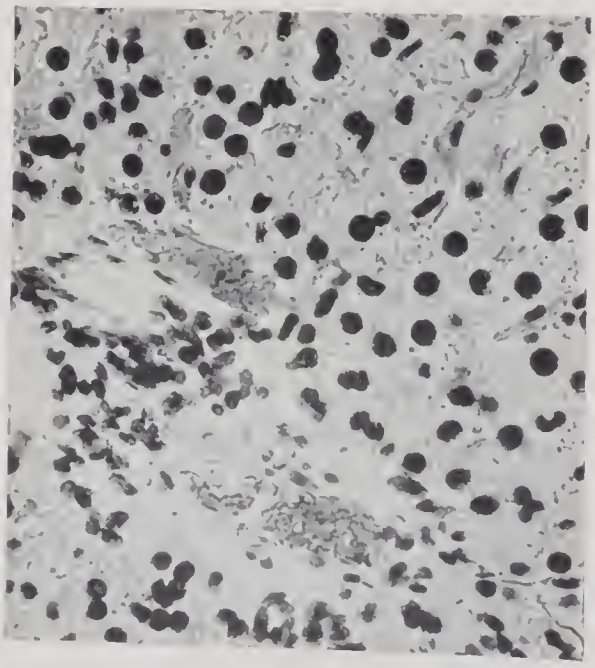


FIG. 132. Frozen section (10 μ) of rat liver after 40 hours' cold formalin fixation and 5 hours in Gomori's (1941) medium. Long fixation produces predominantly nuclear staining. Eosin. × 220.

[*To face p.* 440.

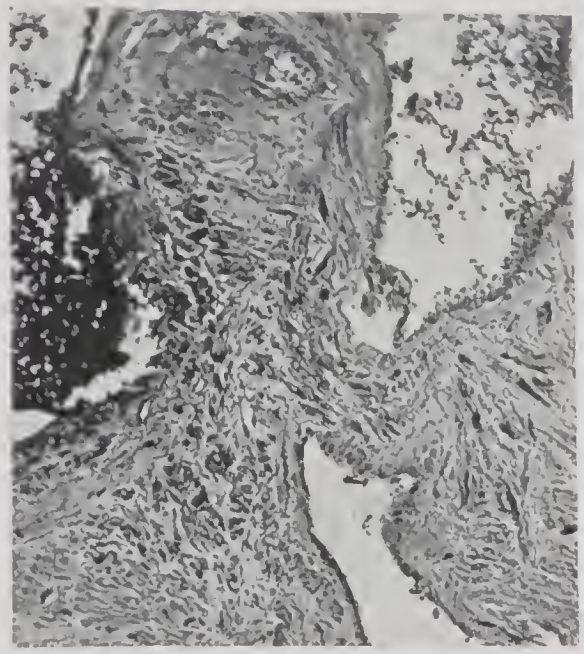


FIG. 133. Paraffin section (5·5 μ) of human prostate. Application of the acid phosphatase (azo dye) technique of Seligman and Manheimer. The dye is largely localized in the secretions in the acinar lumen. × 130.

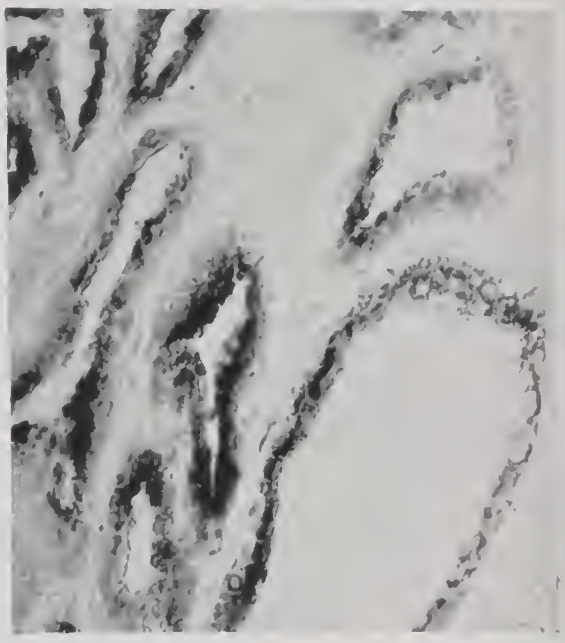


FIG. 134. Frozen section (10 μ) of dog prostate. Incubated for 30 minutes in Gomori's (1950) medium for acid phosphatase. For comparison with Plate IIIc. × 235.

FIG. 135. Rat kidney. Proximal convoluted tubule. Osmotic protection of fresh frozen section with 7·5 per cent. PVP. Acid phosphatase is localized in rod-shaped and ovoid bodies measuring 0·6–1·2 μ. Note absence of background staining. Standard coupling azo dye method. × 4200.

absence of substrate in order to assess the degree of non-specific staining.

Using this azo dye method, Seligman and Manheimer found that the distribution of acid phosphatase in normal human tissues was the same as that reported by other observers using the Gomori method. On the basis of a selective inhibition by D-tartaric acid, following the observations of Abu'l-Fadl and King (1948a and b), they described two varieties of acid phosphatase detectable histochemically. One, the type found in prostatic epithelium, was completely inhibited by 4 per cent. tartaric acid, while the other, present mainly in the nuclei and in skeletal muscle, retained its usual activity. The second variety was completely inhibited by 10 per cent. tartaric acid.

The best results which I was able to obtain with the Seligman-Manheimer method were far from satisfactory and they compared unfavourably with the results of the Gomori (1950) method. Three factors were responsible for these poor results. First, the extremely small amount of acid phosphatase remaining in paraffin sections which made the long incubation period inevitable. This led not only to diffusion of enzyme and reaction product, and to non-specific staining of the background, but also to diffuse precipitation of some of the breakdown produets in particulate form. The general effect, for this reason, was usually dirty. Secondly, the low solubility of calcium α-naphthyl phosphate made it impossible to obtain the substrate concentration necessary for maximum enzyme activity. This factor also prolonged the incubation period. Thirdly, anthraquinone-1-diazonium chloride, though strongly recommended by Seligman and Manheimer (1949), was found quite unsatisfactory as the coupling agent for this technique.

The necessary qualities of a diazonium salt for use in enzyme histochemistry of the type we are considering are : (1) that it should be sufficiently soluble at the pH employed ; (*b*) that it should decompose slowly at this pH ; (*c*) that it should not inhibit the enzyme ; (*d*) that it should couple rapidly with the enzymically released α-naphthol or other phenol ; (*e*) that the resulting azo dye should be absolutely insoluble in the incubating medium ; (*f*) that it should be highly coloured ; and (*g*) that the size of the dye particles should be small. In the case of paraffin sections, an additional desirable quality is that the dye should be insoluble in alcohol and xylene so that permanent preparations may be made. In the case of frozen sections this quality is less necessary, since it is not advisable to mount these in balsam or other permanent medium in view of disturbances caused by the necessary dehydration.

The Standard Monocoupling Technique (Simple Naphthols). Grogg and Pearse (1952), having found that only broad localization of acid

TABLE 32

Properties of Coupling Agents at pH 5·0

(Frozen Sections)

	Diazonium salt of	Inhibition	Decomposition	Colour	Diffuse or particulate
1	1-amino-anthraquinone	++	++++	Brick-red	Diffuse
2	4-benzoylamino-2 : 5-dimethoxyaniline	++	+	Brick-red	Particulate +
3	*p*-nitroaniline	+++	+++	Orange	Particulate +
4	4-chloro-2-nitroaniline	+++	++	Orange-red	Particulate +
5	2 : 5-dichloroaniline	+++	+++	Orange-red	Particulate +
6	*o*-dianisidine	+	++	Dark red	Diffuse
7	4-chloro-*o*-anisidine	++++	+	Red	Particulate
8	5-nitro-*o*-anisidine	++	++	Brown-orange	Particulate +
9	5-chloro-*o*-toluidine	++	+	Dark orange-red	Particulate
10	4-nitro-*o*-anisidine	++	+	Orange-brown	Particulate +
11	3-nitro-*p*-toluidine	+++	+	Orange-red	Particulate +
12	3-nitro-*p*-anisidine	+++	++	Orange-red	Particulate +
13	4-amino-3 : 1′-dimethyl azobenzene	++	+	Red-brown	Particulate +
14	4-amino-2 : 5-dimethoxy-4-nitroazobenzene	+	++	Purplish-red	Particulate
15	4-amino-4′-methoxy diphenylamine	++++	+++	Nil	—
16	3-nitro-*o*-toluidine	+++	+	Nil	—
17	4-amino-4′-nitro-3 : 6-dimethoxy azobenzene	++	+++	Red	Particulate +
18	4-amino-3 : 1′-dimethyl-azobenzene	++	+	Red-brown	Particulate
19	2-methoxy-5-diethylamino-sulphaniline	++	++	Brick red	Particulate
20	2-benzoylamino-4-methoxy-toluidine	+++	+	Red	Particulate +
21	4-amino-diphenylamine	++++	+	Nil	—
22	*p*-*p*′-diaminodiphenylamine	++	+	Brown	Particulate +

In the above Table degrees of inhibition are registered from + to ++++, the former indicating almost complete absence. Similarly, in the case of decomposition, one + means practically no visible staining of the background structures. The reference particulate means that the dye particles were large enough to be clearly visible with the 2/3rds objective ; diffuse means a particle size not appreciable at this magnification.

The addition of + means that the particles were larger than is desirable.

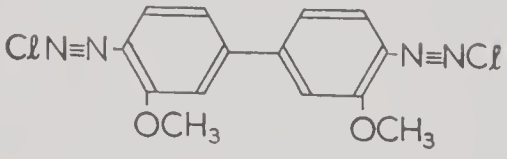
Numbers given are Laboratory Reference Numbers given in the Table of Diazotates in Appendix 14, p. 870. Numbers in bold type are those of recommended salts. Apparently identical salts have different stabilizers.

phosphatase could be obtained by Gomori-type methods, modified the coupling azo dye technique in the light of the above observations. In order to overcome the first objection, cold formalin-fixed frozen sections were substituted for acetone-fixed paraffin sections. The second objection was removed by the substitution of the very soluble sodium α-naphthyl phosphate for the calcium salt, and there remained, therefore, only the selection of a suitable coupling agent for the

precipitation of α-naphthol at pH 5·0. With this object in view, the stable diazotates or bis-diazotates of the above compounds were tested in respect of the above criteria, using sections of human and dog prostate and mouse and rat liver.

With only six of the coupling agents listed was the final result of incubation at all satisfactory. These were salts 6, 7, 9, 14, 18 and 19. The best results were obtained with salt 18 (Fast Garnet GBC salt, I.C.I. Ltd.), which satisfied all the criteria listed above except the additional one of insolubility in alcohol and xylene. This failure was regarded as unimportant since the frozen sections, which were finally used as a routine, were always mounted in glycerine jelly. Sections prepared with salts 18 and 19 are fairly stable. No change in the distribution of the final azo dye product, or in the size of its particles, could be detected after 4 months' storage at room temperature.

Effect of pH on the Behaviour of Diazonium Salts. In the course of these and other investigations into the nature of the coupling azo dye techniques some interesting observations on the behaviour of diazonium and bis-diazonium salts at various pH levels were made. If diazotized *o*-dianisidine, at the optimum concentration, is coupled with enzymically (alkaline phosphatase) released α-naphthol at pH 9·2, the resulting dye is black and insoluble in alcohol and xylene as well as in water. If it is coupled with α-naphthol released enzymically (esterase) at pH 7·4, the dye is at first red and later black, as more is produced. It is only partially soluble in alcohol and xylene. When coupled with α-naphthol released by acid phosphatase at pH 5·0, as in the experiments here described, the dye is purplish-red and soluble in alcohol and xylene. These findings indicate that the mode of coupling in the case of this bis-diazonium salt depends, *inter alia*, on the pH. A possible explanation of the mechanism of the present example is that at higher pH levels both ends of the molecule are able to react, whereas at acid pH levels only one end is available for coupling. In



Diazotized *o*-dianisidine.

this case the bis-diazonium salt acts like many mono-diazonium salts and produces a red dye on coupling with α-naphthol. These observations lead naturally to consideration of Burton's (1954) modification of the Seligman-Manheimer technique.

Pyridine or Quinoline-Catalyzed Coupling. When salt 6 was used in the standard type of coupling method for acid phosphatase, with high substrate concentrations and relatively long incubation periods,

Burton found that the most active sites contained black pigment and the weaker sites the usual red product. When the concentration of the diazonium salt was below 1 mg./ml. the reaction was rapid and the final product always red. With high concentrations of salt 6 the reaction was appreciably slower but the product was predominantly black. Burton therefore introduced the use of catalyzers to increase the coupling rate (see Chapter XIII, p. 370), and for this purpose he employed pyridine or 2-butoxy-N-(2-diethylaminoethyl) cinchonamide hydrochloride (percain). After incubation sections were treated with acid alcohol before dehydration with alcohol, clearing in xylene and mounting in a synthetic medium.

The results of the modification certainly appeared to be much sharper than those obtained by the usual technique with salt 6, in which diffusion was obvious after all but the briefest incubation periods. Unfortunately Burton's technique introduces an entirely new artifact in that the black final dye product is produced only in the most active regions. Elsewhere the dye retains its usual red colour. Furthermore the black dye, considered by Rutenburg and Seligman (1955) to be a polymerized product due to multiple coupling, is insoluble in alcohol and xylene. During the final processing in Burton's technique, therefore, differentiation is produced with the result that only areas of strong activity remain stained. The red dye in the weaker areas is entirely removed. For this reason the technique, as it stands, cannot be recommended.

Results Obtained with the Standard Method. Using this method and incubating frozen sections for periods of up to 1 hour, only cytoplasmic structures were observed to be stained. In the case of human prostate, for instance, the cytoplasm of the acinar cells was strongly stained and diffusion of enzyme during the postmortem interval was indicated by staining of the connective tissues and muscle around the areas of strongest activity. With dog prostate, fixed immediately after removal, an incubation time of $\frac{1}{2}$–1 minute (with salt 6) gave satisfactory results. Here (Plate IVB) only the luminal part of the cytoplasm of the acinar cells was stained purplish-red. Fig. 134 should be compared with the coloured illustration; it was produced by $\frac{1}{2}$-hour incubation in Gomori's (1950) medium and it shows that with this method the rough topographical localization of enzyme activity is the same as by the azo dye method. The intracellular localization, however, is quite different. Staining of the nuclei was never observed to occur when the azo dye technique was applied to formalin-fixed frozen sections, although in paraffin sections some indistinct nuclear staining was occasionally observed. With the Gomori method it was invariably present, even with short incubation periods. The relatively weak acid phosphatase of the red cells, which was shown by Abu'l Fadl and King (1949) to be

destroyed by formalin, gave no positive reaction by the azo dye technique in formalin-fixed sections. In unfixed sections a weak reaction is sometimes observed. Plate IVc shows acid phosphatase in the β-cells of the pancreatic islets, using salt 19 as the diazonium component of the incubating medium.

The standard simultaneous coupling azo dye method gives excellent results, particularly with salt 18 (Fast Garnet GBC), in a variety of different tissue preparations. The latter include cold formol-calcium-fixed, frozen, free-floating sections, fresh unfixed cold microtome sections mounted on coverslips, and the same preparations post-fixed either in cold formalin or in cold acetic-ethanol. Incubation for 15–30 minutes is usually adequate and in the first two types of tissue preparation mentioned above the final dye product appears to be localized in bodies resembling mitochondria or lysosomes (Fig. 135, p. 441). If protective media (containing 7·5 per cent. PVP) are employed the reaction proceeds more slowly but the mitochondrial type of localization is more easily obtained. Although in the past I have recommended and used cold formalin-fixed frozen sections for studies on acid phosphatases, those who have read some of the preceding chapters of this book will have realized that I now consider the use of unfixed cold microtome sections essential for most methods in enzyme histochemistry. This particularly applies when one is dealing with labile enzymes like acid phosphatase. Only if one is familiar with the results obtained in preparations of this type is it permissible to work with sections treated in other ways in which the percentage of surviving enzyme may be small, and confined to one only of the types present in living tissues.

Monocoupling Techniques (Substituted Naphthols)

Five mono- and dihalogen-substituted naphthylphosphates (6-bromo-2-naphthyl ; 1-chloro-2-naphthyl ; 1,6-dibromo-2-naphthyl ; 2,4-dichloro-1-naphthyl ; 2,4-dibromo-1-naphthyl) were tested by Pearse (1954) as substrates in simultaneous coupling azo dye methods for both acid and alkaline phosphatases. The results obtained at pH 5·0 with 6-bromo-2-naphthyl phosphate were similar to those obtained with α-naphthyl phosphate, but with the two dihalogen-substituted α-naphthols the results were certainly superior. Although according to Fierz-David and Blangey (1949) diazonium coupling cannot take place with α-naphthol derivatives substituted in the 2 and 4 positions it was observed to take place readily under histochemical conditions or in the presence of pyridine or alcohol. Presumably under suitable conditions the ejection of one of the halogen groups allows coupling to take place.

Gomori (1956a) also tested 2,4-dichloro- and 2,4-dibromo-1-

naphthyl phosphates as substrates for acid phosphatase and found that the results obtained were "essentially the same as, although æsthetically far inferior to, those obtained by the Rutenburg-Seligman method". This method is described below on p. 447. According to Gomori (1956b) the molecular extinction of azo dyes from the two halogenated α-naphthols is less than one-fifth that of the dyes from 6-bromo- or 6-benzoyl-2-naphthol. This is a powerful objection to their use in histochemistry unless evidence in their favour can be produced on other counts.

An alternative approach to the problem is that of Burstone (1958), who carried out studies similar to those reported in the previous chapter (p. 408) using a variety of substituted Naphthol AS phosphates. In conjunction with these Burstone particularly recommended the use of freeze-dried sections although cold-acetone-fixed paraffin sections and cold formalin-fixed frozen sections were stated to give good results. As substrates the phosphates of Naphthol AS-BI, AS-TR and AS-MX were found to be stable at acid pH levels and readily hydrolyzed. Long incubation periods are perfectly feasible and suitable diazotates are salts 4 and 5 (p. 870) and Fast red-violet LB.

The advantages of substituted naphthoic arylides as reaction products are due to three things. First, their insolubility in aqueous media; secondly, their substantivity for protein; and thirdly, the high molecular extinctions of azo dyes derived from them. From Table 33 it can be seen that the solubility in water of Naphthol AS and its derivatives is far less than that of any other substituted or unsubstituted naphthols.

TABLE 33

Solubilities of Naphthols (after Gomori (1956b) and Burstone)

(0·05 M-acetate; pH 5·0; 22°)

Naphthol	Solubility (micrograms/ml.)
2-Naphthol	740
1-Naphthol	303
6-Bromo-2-naphthol	195
6-Benzoyl-2-naphthol	9–27
2,4-Dichloro-1-naphthol	12
2,4-Dibromo-1-naphthol	3·5
Naphthol AS and derivatives	< 1

The results of the application of Burstone's method, details of which are given on p. 882 (Appendix 15), are æsthetically very satisfactory, especially in freeze-dried sections. Nuclear staining is never observed

and in rat liver, for instance, the chief activity is localized in the bile canaliculi (Fig. 136, p. 448). A faint diffuse colour is also evident throughout the cytoplasm of the liver cells. These appearances suggest that predominantly only one of the acid phosphomonoesterases is being demonstrated. In rat pancreas there is strong activity in the β-cells of the islets, but the degree of localization is no better than with the standard α-naphthyl phosphate method.

Post-coupling Techniques for Acid Phosphatases

The demonstration of acid phosphatase by a post-incubation coupling technique using 6-bromo-2-naphthyl phosphate as substrate was proposed by Seligman *et al.* (1949). The method proved unsatisfactory, however, because of the long incubation period required and the solubility of 6-bromo-2-naphthol (see Table 33). My own observations on post-coupling techniques based on esters or glycosides of this naphthol (Pearse, 1954 ; Burton and Pearse, 1952) caused me to regard the post-coupling techniques in general as unreliable indicators of enzyme localization. Improvements in the post-coupling technique for acid phosphatase developed by Rutenburg and Seligman (1955) necessitate some revision of this opinion.

The newer method is based on the use of sodium 6-benzoyl-2-naphthyl phosphate as substrate, and details of its preparation are given in Appendix 15. As described, the original incubating medium was made hypertonic with 2 per cent. NaCl with the idea of preventing diffusion of enzyme during incubation. The same result is perhaps better achieved by the use of PVP (Scarpelli and Pearse, 1958) or methyl cellulose (Hannibal and Nachlas, 1959). Formalin-fixed free-floating sections were originally recommended, but fresh-frozen sections mounted on slides were also used. After relatively short incubation periods (½–2 hours) the sections were washed in cold saline and then treated with Fast blue B salt at pH 7 to 8. In active tissues the final product was a deep blue azo dye (Fig. 137), but when dissolved in lipid tissue components this appeared purplish-red. Weaker areas of activity stained red in any case (monocoupling). If this spectrum of colours is considered undesirable, Fast Garnet GBC or red-violet LB salts can be used instead of Fast blue B salt.

The relative insolubility and high substantivity of the primary reaction product, which reduce its diffusion to a very low figure, make diffusion of enzyme the most important limiting factor in accurate localization. Provided that the incubation period can be kept short (with active tissues it may be as low as 1 minute) the results of the application of the method to fixed frozen sections are excellent (see Fig. 137, p. 448). I have not been able to obtain mitochondrial or lysosomal localization of enzyme with the method, however, and the

preparations are not stable for much over 14 days. The main advantage of the post-coupling method is that it allows the shortest incubation times to be used since there is no inactivation of enzyme by diazonium salt as there is in the simultaneous coupling methods.

The view was expressed by Gomori (1956a) that the post-coupling technique was the best available at that time for the general demonstration of acid phosphatases. With the advent of simultaneous coupling methods using Naphthol AS phosphates this view may have to be modified. In making a choice between several available methods it is necessary here, as elsewhere, to ask oneself what it is that one requires. I have been able to obtain precise mitochondrial or lysosomal localization of acid phosphatase, in fresh frozen sections, using the standard azo dye method (see Fig. 135, p. 441). On the other hand, in paraffin sections the results are far more satisfactory with Burstone's method. In formalin-fixed frozen sections the best overall result is obtainable with the post-coupling technique. At the time of writing no comprehensive comparative study of the available methods for acid phosphatase has been made, and until the results of such a study are available it is necessary to consider each of the methods and to choose the one best suited to the problem in hand.

Comparison with Biochemical Methods. It is interesting to compare the intracellular localization of acid phosphatase demonstrated by the various azo dye methods with that obtained by means of techniques in the biochemical field. Palade (1951) compared the results of his determinations of acid phosphatase in rat liver homogenates, and in various nuclear and cytoplasmic fractions isolated therefrom, with the lead phosphate methods (1941 and 1950) of Gomori. With the biochemical technique the enzyme was found to be almost entirely (95 per cent.) cytoplasmic and the author interpreted the 5 per cent. found in the nuclear fraction of his homogenates as being entirely due to contamination. With both Gomori's techniques the enzyme was predominantly concentrated in the nuclei. Similar observations were made by Berthet and de Duve (1951) and by Berthet *et al.* (1951). In the first-mentioned paper the authors described the separation from rat liver homogenates of a mitochondrial fraction containing 55–60 per cent. of the total acid phosphatase. The remainder was found partly in smaller granules and partly in the supernatant, while the nuclear fraction contained only a low percentage of the total activity.

The older azo dye methods gave results which agreed with those of Palade in placing 100 per cent. of the non-specific acid phosphatase of rat liver in the cytoplasm, and they indicated that the enzyme was usually distributed evenly throughout the cell, not concentrated along the bile canaliculi as it sometimes is with the Gomori method.

A further finding of Berthet and de Duve may be of importance in

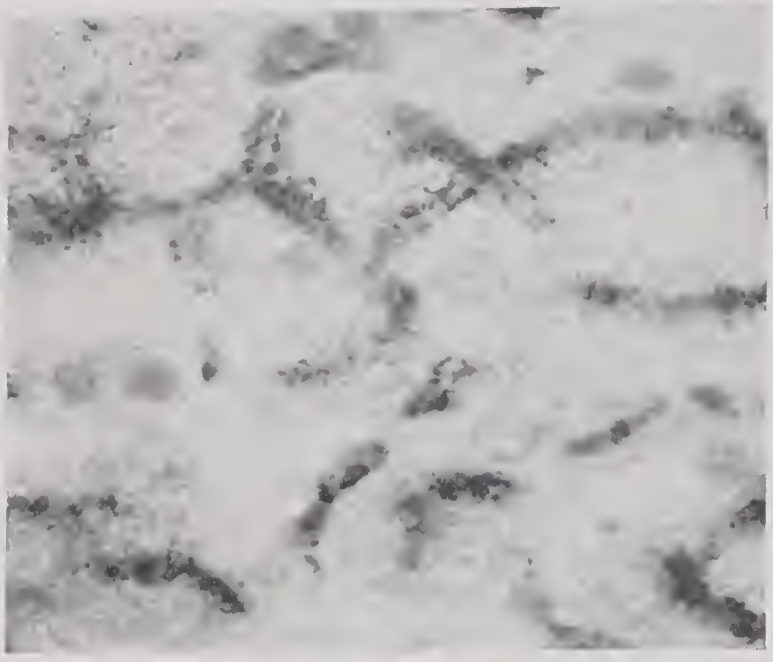


FIG. 136. Rat liver. Paraffin section. Shows surviving acid phosphatase in proximity to bile canaliculi. Burstone's Naphthol AS-BI method. × 800.

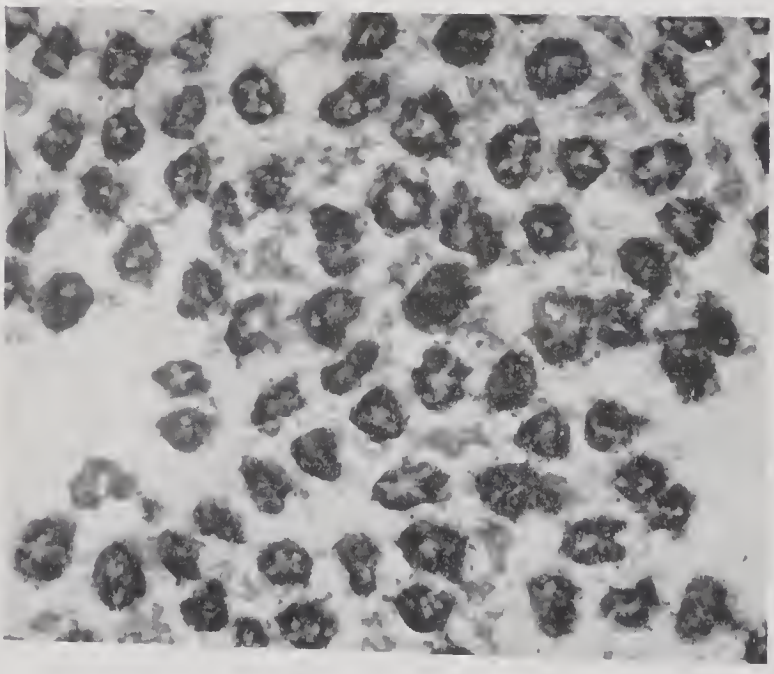


FIG. 137. Rat kidney. Acid phosphatase in tubules. Post-coupling method. × 200.

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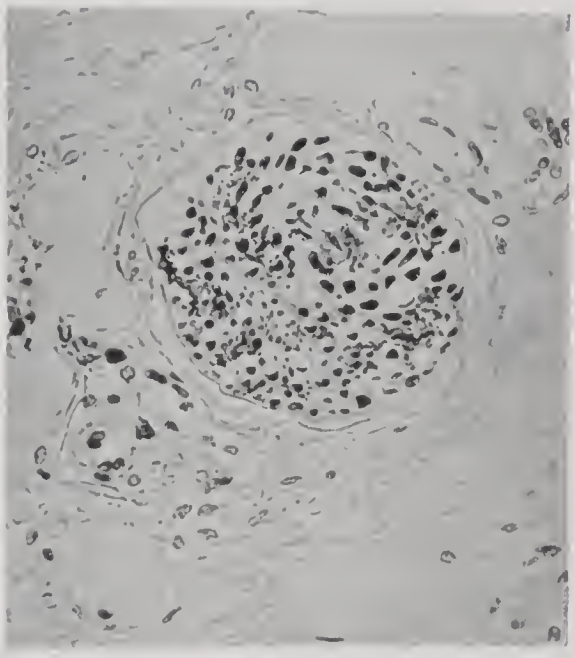


FIG. 138. Cold acetone-fixed paraffin section. Showing localization of phosphamidase, especially in the nuclei and axons of a small medullated nerve. Phosphamidase method. × 205.

relation to histochemical techniques for acid phosphatase. When these authors compared the activity of their original homogenates with the total activity of the fractions isolated therefrom, an increase of 100 per cent. was found. This was attributed to the existence of a large proportion of the enzyme in a bound form which was inactive towards glycerophosphate. Activation was produced by several manœuvres, including ageing at 0° and repeated freezing and thawing. It may be supposed that cold formalin fixation, and the freezing and thawing incurred in the preparation of frozen sections, is effective in releasing the bound form of the enzyme. Such a mechanism may explain the finding by Grogg and Pearse (1952) of a lesser acid phosphatase activity in freeze-dried by comparison with formalin-fixed sections of the same dog prostate, as judged by the amount of colour developed in the same time by a coupling azo dye technique.

Continuing their studies on rat liver homogenates, de Duve and his associates (Appelmans *et al.*, 1955 ; de Duve *et al.*, 1955) have described a special class of intracytoplasmic organelles which they call lysosomes. The acid phosphatase of rat liver is localized especially in small particles consisting of lysosomes and small mitochondria. Using the improved standard simultaneous coupling technique and thin, protected, cold microtome sections Scarpelli and Pearse (1958) have shown that acid phosphatase can be localized in short rods and ovoid structures measuring 0·5–1·2 μ (Fig. 135, p. 441), and these are considered to represent the lysosomes.

Results of this order are not required in routine applied histochemistry, whether this be diagnostic or comparative, and possibly they will not be required even for quantitative studies. For critical studies of the intracellular distribution of acid phosphatases, however, especially if these are to be combined with biochemical investigations, methods which afford a fine degree of localization are to be regarded as essential.

Techniques for Specific Acid Phosphomonoesterases

Lead Nitrate Technique for Phosphoamidase (DW. 88)

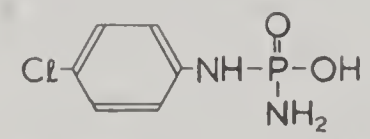
In the course of experiments on the histochemical specificity of phosphatases, identical patterns of enzyme distribution were found with eighteen out of the nineteen substrates employed by Gomori (1949). The nineteenth substrate, described as *p*-chloranilidophosphonic acid, yielded a pattern identical with the others at pH 9 and 7 but gave very different pictures at pH 5. This finding strongly favoured the existence of a specific enzyme and a method for its demonstration was, therefore, set forth by Gomori (1948).

A specific enzyme acting on phosphocreatine was first described

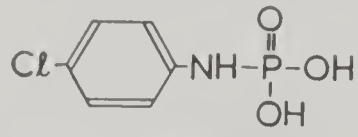
by Waldschmidt-Leitz and Köhler (1933) and by Ichihara (1933), and later by Bredereck and Geyer (1938). It was present in a wide variety of animal and plant tissues. Holter and Li (1950, 1951) showed that in brain extracts the enzyme had a pH optimum of 4·6 and that activity declined rapidly on either side of this point. They also showed (1950b) that *p*-chloranilidophosphoric acid monoamide was hydrolyzed by trypsin, pepsin and chymotrypsin. Several points of distinction between phosphoamidase and non-specific acid phosphatase were found and there seems to be no doubt of the existence of the specific enzyme. Speculations about the possible functions of phosphoamidase in brain were made by Tseitlin (1952), who found a high specific activity using phospholysine as substrate and later (1953), using phosphocreatine.

It was shown by Rorig (1949) that the method used by Gomori for the synthesis of his substrate did not, in fact, yield *p*-chloranilidophosphonic acid but N-*p*-chlorophenyldiamidophosphonic acid.

The first of these two compounds can be synthesized, however, by a method given by Li (1950).



p-chloranilidophosphonic acid.



N-*p*-chlorophenyldiamidophosphonic acid.

The above disclosure does not in any way invalidate Gomori's experimental observations, and these are described below. Acetone-fixed paraffin sections were used and these were incubated with 0·004 M substrate between pH 5·4 and 5·8, in the presence of 0·001 M-lead nitrate. A 0·05 M-maleate buffer was employed and manganese ions as activator. Above pH 6·2 the reaction became weaker and above pH 6·7 it vanished. Below pH 5·3 artifacts due to lead impregnation of tissues were numerous. They could be distinguished by examination of slides inactivated by treatment with Lugol's iodine for 5 minutes. Many other drawbacks were present ; at pH 5·6, for instance, the substrate was not entirely stable and it was found necessary to incubate the sections face downward or facing downwards at least. After incubation a good deal of the superficial precipitate was removed by treatment with 0·1 M-citrate buffer at pH 4·5 – 5, followed by washing in water, and the sites of enzymatic activity were demonstrated by conversion of the lead salt to its sulphide by yellow ammonium sulphide.

Gomori did not find the method to be entirely dependable in that uniformity of results was not achieved. In two tissues particularly, however, a strong reaction invariably occurred. These were the grey matter of the central nervous system (especially the cerebellum) and malignant epithelial tumours. In the latter, the intracellular localization of the enzyme was in striking contrast to the extracellular situation of alkaline phosphatase in the majority of cases. I applied the phosphamidase technique to a variety of tissues fixed in cold acetone and embedded in paraffin, following the exact directions of Gomori. In the case of the majority of tissues a satisfactory result could not be obtained with less than 16–24 hours' incubation. By employing standard 16-hour cold-formalin-fixed frozen sections this time was reduced to 2–4 hours. Fig. 138 shows the result of applying the method to paraffin sections and it indicates the predominant localization of precipitate in the nuclei and in nervous tissues, in a medullated nerve fibre in this example.

The majority of workers who employed the original technique of Gomori obtained unsatisfactory results and it was usually criticized even by those (e.g. Meyer and Weinmann, 1953) who employed it with a certain degree of success. These authors used the original technique on cold ethanol-fixed paraffin sections as did Winter (1954–55) in his studies of the normal and neoplastic human cervix uteri. Eger and Schulte (1954–55), on the other hand, used fresh-frozen sections in their study of the enzyme in rat liver and kidney. The localization observed was in both cases different from that of acid or alkaline phosphatase and the authors concluded that they were dealing with a specific enzyme. There is no doubt that the localization in fresh tissues is sometimes different from that of acid phosphatase and my earlier observations in the first edition of this book must be withdrawn. The distinction between phosphoamidase and acid phosphomonoesterase activity still rests largely on localization differences, however, and these have often been invented where they do not in fact occur. For instance, Meyer and Weinmann (1957) give a list of sites in the rat containing high phosphoamidase activity from which, according to their researches in the literature, acid phosphatase is absent. These include the granular cells of keratinizing epithelium, gastric parietal cells, secretory cells of the seminal vesicle, adrenal cortex and pancreatic islet (β-) cells. All these according to my observations possess a strong or very strong acid phosphatase with almost maximal activity at pH 5·2 (and see Rutenburg and Seligman, 1955). While I am prepared to admit that the lead nitrate technique may show a separate and specific phosphoamidase, proof of this fact is not vouchsafed by inaccurate assertions like those reported above.

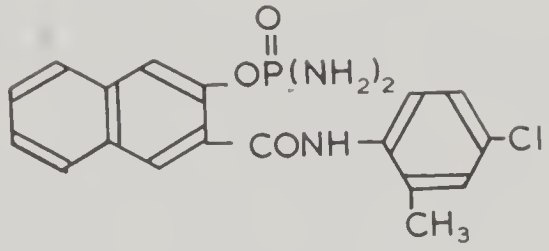
Notwithstanding these criticisms, it must be acknowledged that

Meyer and Weinmann (1955) have produced a distinct improvement on the original method of Gomori. I have tested their method with fresh frozen and formalin-fixed frozen sections as well as with the conventional paraffin sections, and I conclude that the use of the latter is obligatory if worthwhile results are to be obtained. The modified method appears in Appendix 15, p. 884. It is, in my opinion, still too capricious for routine application to pathological and other problems.

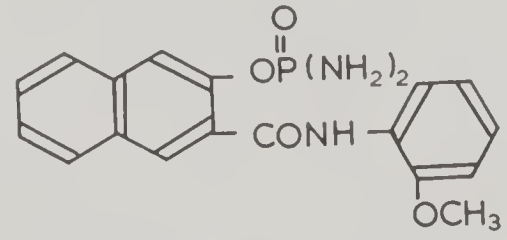
Coupling Azo Dye Methods for Phosphoamidase

Histochemical attempts to demonstrate the hydrolysis of phosphoamides by capture of the alcoholic reaction product have hitherto been delayed by lack of appropriate naphtholic substrates. Preliminary reports by Burstone (1958) suggest that the phosphoamides of substituted AS-naphthols which he has synthesized may be useful substrates for a specific phosphoamidase. The two most promising compounds are the phosphoamides of Naphthol AS-TR (I) and AS-BI (II).

I



II



Details of the synthesis of Naphthol AS-phosphoamides are given in Appendix 15, p. 885. These substrates are used in the same way as Burstone's substrates for acid phosphatase.

Lead-Nitrate Technique for Deoxyribonuclease II (DW. 124)

This mainly mitochondrial enzyme breaks down desoxyribonucleate with the formation of polynucleotides (purine and pyrimidine-3′-nucleotides). The pH optimum of the enzyme is between 4·5 and 6, and Mg^{2+} ions do not, in this case, act as activators. An attempt to demonstrate the enzyme histochemically has been made by Aronson *et al.* (1958), using a Gomori type lead nitrate procedure. In order to produce a reaction product capable of capture by Pb^{2+} ions the activity of other enzymes is necessary. The nucleotide reaction product of the first part of the reaction is presumed to be acted on by endogenous

non-specific acid phosphatase and by 5-nucleotidase. The authors added exogenous acid phosphatase to their medium but could not obtain any evidence that it was effective. Reliance on endogenous auxiliary enzymes obviously constitutes a weakness of the method since the final localization of precipitate can at best only be that of the auxiliary enzyme. There is something of a parallel here to the dehydrogenase-diaphorase problem (Chapter XIX, p. 555), in which the localization of the dehydrogenase is inevitably that of its associated diaphorase. If the separation between the two is only a separation at the molecular level theoretical objections are of little practical importance.

Aronson and his co-workers use three control sections, one lacking substrate, one lacking exogenous acid phosphatase and the third containing 0·01 M-NaF. The method suffers at present from all the defects of the original method and a few extra ones of its own. Nevertheless the authors believe that absence of staining in fluoride treated sections supports the idea that acid phosphatase, acting as a non-specific nucleotidase, is responsible for production of orthophosphate, and thus for the final precipitate. This method requires further testing.

Digestion Method for Deoxyribonuclease

An ingenious method for the histochemical demonstration of enzymes of this class was suggested by Daoust (1955, 1957). Films of gelatine containing DNA are applied to microscope slides and fixed *in situ* with formalin. Fresh frozen sections are then mounted on the films and incubated in a moist chamber for 5 hours at 20°. After fixation of the tissue, performance of the Feulgen reaction (Chapter VIII, p. 193) colours the nuclei in the section and also the underlying film of DNA magenta. Negative (unstained) images in the stained sheet of DNA indicate sites of DNAse activity.

Localization with methods of this type is necessarily crude. Although the method of Aronson *et al.* produces a sharper image it does not necessarily afford a more accurate localization of enzyme. The two methods should be compared.

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CHAPTER XVI

CARBOXYLIC ESTERASES

In Chapters XIV and XV those particular esterases which hydrolyse phosphate esters have been dealt with. There remain to be described in this chapter a group of esterases, of probably diverse function *in vivo*, whose common link is that their substrates are esters of carboxylic acids. Out of the 17 enzymes in this group listed by Dixon and Webb (1958) histochemical techniques exist for four. These are the so-called ali-esterases, the lipases, acetylcholinesterase and pseudo-cholinesterase. In addition, it is possible to demonstrate certain other non-specific esterases which have been classified biochemically, and an additional esterase which has not been so described.

Ali-esterases, Arom-esterases and Lipases

In dealing with these three classes of enzyme we are dealing with a large and widespread group acting at optima between pH 5 and 9 on a variety of substrates. All these enzymes catalyse reactions of the type

$$RCOOR' + H_2O \rightleftharpoons RCOOH + R'OH$$

and the majority are both hydrolytic and synthetic in action. If a simple ester such as methylbutyrate or naphthyl acetate is the substrate concerned, the enzyme is classed as a *non-specific esterase*. The non-specific esterases can be broken down into several different types, of which the *ali-esterases* and the *arom-esterases* are the most important histochemically. Recently a group of *acetylesterases* has been separated from the non-specific esterases whose members hydrolyse esters of acetic acid with particular efficiency. If the substrate is the ester of a higher fatty acid with glycerol or some other alcohol, the enzyme concerned is a *lipase*. The esterases, however, also act on fatty acid esters so that there is some degree of overlap between the two.

Distinction between Lipases and Esterases (Historical Methods)

As early as 1906 Loevenhart distinguished between pancreatic "lipase" and liver "esterase," but despite this the terms lipase and esterase have continued to be used interchangeably. Broadly speaking, the esters of short-chained fatty acids (C_2—C_4) are acted upon by esterases, and the long-chained esters (C_8 and upwards) by lipases. A very large number of studies have been made, using various fatty acid esters and various chemical methods of estimation, on the

differences between these enzymes. The differences recorded include substrate-specificity, stereochemical specificity, behaviour towards activators and inhibitors, and pH optima. Gomori (1948) concluded that owing to multiple overlapping of differences and similarities it could not be decided whether the various effects were due to several well-defined individual enzymes or to the presence of unidentified accompanying substances. Seligman and Nachlas (1950), however, surveying the methods used for measuring the esterolytic activity of serum, declared that at least two distinct esterolytic enzymes existed ; these were esterase and lipase. The former, abundant in liver, kidney, serum and pancreas, acted preferentially on short-chained fatty acids and was partially inhibited by sodium taurocholate. Lipase, which was abundant almost exclusively in pancreas, acted preferentially on esters of long-chained fatty acids and was activated by taurocholate. This activation of the true lipases by salts of the bile acids is characteristic, and they have also been shown to be activated by amino-acids and by peptides such as leucylglycylglycine, for instance (Dawson, 1927). Esterases are also activated by amino-acids (particularly arginine, lysine and histidine) and peptides. Although a large number of inhibitors of esterases and lipases have been described (e.g. strychnine, quinine, atoxyl), the differential inhibition caused by these compounds is not sufficient to allow their use for distinguishing between the two enzymes histochemically.

Biochemistry of Non-specific Esterases and Lipases

Until comparatively recently biochemical information about the substrate specificity of the esterases has been scanty and inadequate. Fodor (1950) suggested that the hydrolysis of methyl esters such as methyl butyrate was due largely to cholesterol esterase, an enzyme which was distinguished from lipase by Kelsey (1939). The methods used by Fodor were not adequate, however, to permit the definition of relative substrate specificities. A great advance was made by Webb (1948) with the suggestion that the so-called organophosphorus compounds could be used to distinguish lipases from esterases. This work was continued and extended by Aldridge (1953a and b, 1954), by Mounter and Whittaker (1953) and by Myers and his associates (1955a and b), with the result that we can now distinguish at least three separate non-specific esterases as well as a lipase of pancreatic type.

The older methods of distinguishing esterase from lipase activity were found by Myers *et al.* (1955b) to be completely without value, although high concentrations of atoxyl were found to inhibit esterase activity (phenyl butyrate) without affecting lipase activity (tributyrin). Quinine, on the other hand, was found to inhibit both enzymes equally.

The work of Aldridge (1954) has made it clear that the use of a variety of substrates (as in most of the older work) is far less useful in distinguishing types of esterase than the employment of activators and inhibitors.

At the present time the most important inhibitor in the esterase field is E 600 (diethyl *p*-nitrophenyl phosphate) and with the aid of this compound Aldridge (1953a and b, 1954) has distinguished two basic types of carboxylic esterase. These he has called A-esterases and B-esterases. The former, which are not inhibited by concentrations of E 600 up to 10^{-3}M, hydrolyse acetates at a faster rate than butyrates. The B-esterases, on the other hand, are inhibited by concentrations of E 600 as low as 10^{-8}M and they hydrolyse butyrates at an equal rate, or faster than acetates. Myers *et al.* (1955b) have shown that 3×10^{-4}M–E 600, preincubated with suspensions of rat pancreas, inhibits 95 per cent. of the activity of lipase on tributyrin. In his second paper (1953b) Aldridge showed that his A-esterase was identical with the previously recorded E 600-esterase which is capable of hydrolysing a variety of organophosphorus compounds.

Terminology. Some authors (e.g. Hobbiger, 1957) prefer to use the terms organophosphate-resistant and organophosphate-sensitive while others continue to use the term ali-esterase (aliphatic esterase) in conjunction with Aldridge's term arom-esterase (aromatic esterase). Others, yet again, retain the use of specific substrate nomenclature as in the DFP-ase of Mounter *et al.* (1957). The following terms are therefore *approximately* synonymous :

Arom-esterase = Organophosphate-resistant esterase = E 600-esterase = DFP-ase = A-esterase.
Ali-esterase = Organophosphate-sensitive esterase = B-esterase.

These esterases are distinguished from the cholinesterases (see below) by their insensitivity to eserine.

Histochemists will probably prefer the convenient terms A- and B-esterase and, provided it is recognized that these terms may include a variety of similar but not necessarily identical enzymes, there can be little objection to such usage.

Activity of the A-esterases is believed to be bound up with the presence of an SH group in the active centre since these enzymes are sensitive to heavy metals, particularly Hg^{2+}, Ni^{2+} and Cu^{2+}. They are also inhibited to some extent by iodoacetate (Aldridge, 1953b). It is assumed by Bergmann, Segal and Rimon (1957) that the active centre of A-esterase also contains an imidazole ring since the pH activity curves for the hydrolysis of E 600 by rabbit serum A-esterase and of DFP (di-*iso*propyl fluorophosphate) by hog kidney show a maximum at pH 7·6. The B-esterases are also considered to contain an imidazole

ring in their active centre (Wilson and Bergmann, 1950) and probably also serine (Schaffer *et al.*, 1953, 1954).

Recently Bergmann *et al.* (1957) and Bergmann and Rimon (1958) have described a third type of esterase in hog kidney extracts which is resistant to organophosphorus compounds but which does not hydrolyse them. This enzyme they call C-esterase. It is inhibited by Hg^{2+} ions over a wide range of substrate concentrations whereas DFP-ase exhibits a narrow zone of activation. This is shown in Fig. 139, below.

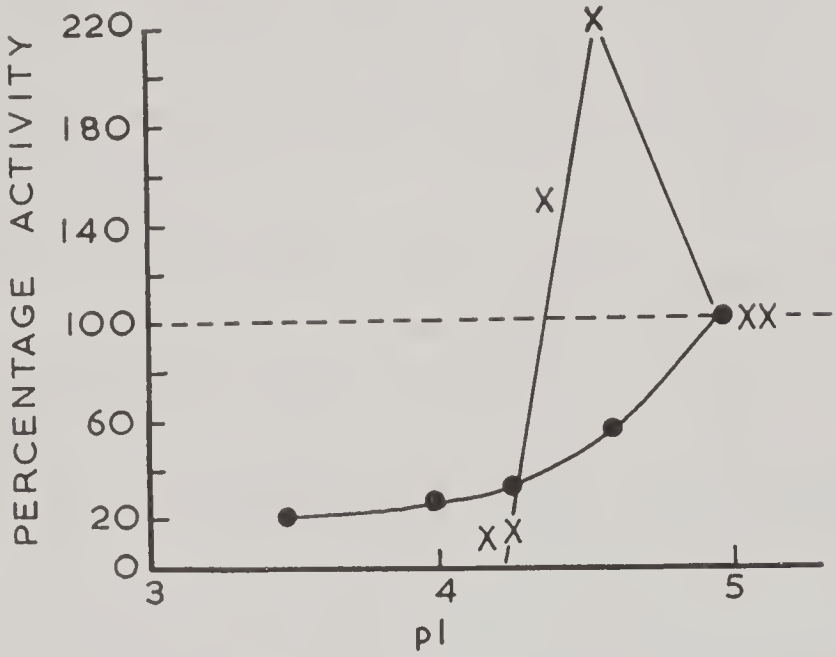


Fig. 139. Effect of Hg^{2+} ions on C-esterase and DFP-ase
● = C-esterase. x = DFP-ase
pI = −log inhibitor concentration (M)

Biochemistry of the Cholinesterases

These enzymes are separated from the other two groups of esterases on account of their ability to hydrolyse esters of choline. Many classifications exist for this group and the most satisfactory is probably that of Augustinsson and Nachmansohn (1949). These authors distinguished the true, or acetyl cholinesterase (AChE) from non-specific, or pseudocholinesterase (ChE). The former has an affinity for its physiological substrate, acetylcholine, higher than for any other ester (except acetylthiocholine) ; it is capable of splitting acetyl-β-methylcholine but not benzoylcholine, and is inhibited by high concentrations of acetylcholine. Non-specific cholinesterase, on the other hand, hydrolyses acetylcholine more rapidly as the concentration of the latter rises and it splits long-chained choline esters at a greater velocity than acetylcholine. It can hydrolyse benzoylcholine but not acetyl-β-methylcholine (Mendel, Mundell and Rudney, 1943 ; Nachmansohn and Rothenberg, 1945).

It is now assumed, therefore, that various types of choline ester-splitting enzymes exist and various methods have been worked out for their distinction *in vitro*. One method is based on the observation that acetyl-β-methylcholine is split only by AChE and another on the fact that butyrylcholine is hydrolysed only by ChE. The selective inhibition of either AChE or ChE by various compounds has also been used for distinguishing the two types. The use of both selective substrates and of selective inhibitors has been applied to the histochemical distinction between AChE and ChE but the position, as will be seen, is complicated by the fact that unnatural substrates must be employed and in many cases these have been inadequately tested *in vitro*. The pH optima of the two enzymes scarcely differ, that of AChE is 7·5–8·0, while ChE is most active between 8·0 and 8·5.

Inhibitors of the Cholinesterases. These substances, which are exceedingly numerous, can be divided into three groups : (*a*) quaternary ammonium bases ; (*b*) physostigmine and urethans ; (*c*) organic phosphorus compounds. Comprehensive lists of substances in the first two groups may be found in papers by Ammon (1943) and Augustinsson (1948), and of those in the last group in papers by Whittaker (1951) and Holmstedt (1951). Burgen and Hobbiger (1951) discussed particularly the organic phosphate inhibitors and Adams and Thompson (1948) gave a list of inhibitors in all three groups. Classical examples in the first group are Nu-1250 (N-*p*-chlorophenyl-N-methylcarbamate of *M*-hydroxyphenyl-trimethyl ammonium bromide) and Nu-683 (dimethylcarbamate of 2-hydroxyphenyl-benzyl-trimethylammonium bromide). In the second group is eserine (physostigmine) itself, and in the third group DFP (di*iso*propyl fluorophosphonate). This compound was at one time extensively used to distinguish between true and pseudocholinesterases since it was found that, in some species at least, the latter were inhibited at a much lower concentration (Mazur and Bodansky, 1946 ; Mendel and Hawkins, 1947 ; Adams and Thompson, 1948). In other species, however (Ord and Thompson, 1950), this difference was not observed. Evidence was produced by Aldridge and Davison (1952a and b) which suggested that organophosphorus inhibitors could be hydrolysed by cholinesterase and Aldridge (1953a) pointed out that a comparison of the sensitivities of true and pseudocholinesterase, in an unpurified condition, was difficult to assess. This was partly due to the presence in tissues or serum of enzymes hydrolysing the inhibitor and this effect is, of course, inevitable under histochemical conditions. Using the specific substrates (acetyl-β-methylcholine and benzoylcholine) for the two enzymes Aldridge (1953c) tested the effect of various inhibitors on their activities. His results appear in Table 34, below.

TABLE 34

Inhibition of AChE and ChE of Horse Blood by Various Inhibitors

Inhibitor	pI_{50}*		Inhibitor Ratio
	ChE	AChE	(AChE/ChE)
Dimethyl *p*-nitrophenyl phosphate	6·10	6·27	0·67
Diethyl *p*-nitrophenyl phosphate (E600)	6·85	6·38	2·9
Di*iso*propyl *p*-nitrophenyl phosphate (DINP)	6·50	5·50	10
Tetraethyl pyrophosphate (TEPP)	8·39	6·52	73
Tetra*iso*propyl pyrophosphate	8·68	5·96	520
Di*iso*propyl fluorophosphonate	8·18	5·75	270
Diethyl fluorophosphonate	7·80	6·09	52
Bisdimethylamino fluorophosphine oxide	3·31	1·96	22
Bismono*iso*propylamino fluorophosphine oxide	7·42	3·82	3,950
Tetra*iso*propylpyrophosphoramide (*iso*-OMPA)	6·48	2·51	9,400

* $pI_{50} = {}^{-}\log_{10}$M concentration of inhibitor to produce 50 per cent. inhibition.

The highest inhibitor ratio was obtained with the compound *iso*-OMPA and this compound was therefore further tested against AChE (erythrocyte) and ChE (plasma or serum) in a number of different animals. In the case of the rat, brain and heart muscle homogenates were used instead of red cells and serum. The results of this study are given in Table 35.

TABLE 35

Sensitivity of AChE and ChE of Various Species to iso*OMPA*

Species	pI_{50}		Inhibitor Ratio
	AChE	ChE	(AChE/ChE)
Horse	2·47	6·52	11,300
Man	3·52	5·27	56
Guinea-pig	2·34	5·89	3,600
Dog	2·42	6·54	13,200
Rat	3·60	6·32	530

It is evident from this table that considerable caution must be used in employing even highly efficient selective inhibitors like *iso*-OMPA in conjunction with histochemical methods for cholinesterases. Any

compound whose inhibitor ratio exceeds 500, however, may be considered as a possible inhibitor for histochemical studies (see below).

Another important study on the use of selective inhibitors for cholinesterases is that of Bayliss and Todrick (1956). These authors tested a number of compounds effective against either AChE or ChE, using homogenates of rat brain or intestinal mucosa and 0·01M-acetyl-β-methylcholine or 0·014M-benzoylcholine as substrates for the respective enzymes. The three selective inhibitors for AChE were Nu-1250 (Roche), 1 : 5-bis-(4-trimethylammoniumphenyl)pentan-3-one diiodide (62C47, Wellcome) which was originally described by Burgen (1948) and by Copp (1953), and 1 : 5-bis-(4-allyl dimethylammoniumphenyl) pentan-3-one diiodide (284C51, Wellcome) described by Copp (1953) and also by Austin and Berry (1953). In the case of ChE only two compounds were tested. These were the irreversible inhibitor tetra-*iso*propylpyrophosphoramide (*iso*-OMPA) and the reversible inhibitor ethopropazine methosulphate (lysivane methosulphate, May & Baker). The results of this study are sumarized in the Table below.

TABLE 36

Inhibition of AChE and ChE of Rat Brain and Intestine by Selective Inhibitors

Inhibitor	Concn. (M)	Percentage Inhibition AChE	Percentage Inhibition ChE
Nu-1250	5×10^{-7}	81	1
	2×10^{-6}	98	8
62C47	5×10^{-6}	94	1
	2×10^{-5}	101	3
284C51	3×10^{-5}	100	2
	1×10^{-4}	102	5
Lysivane	3×10^{-5}	−1	90
	8×10^{-5}	9	95
iso-OMPA	1×10^{-5}	0	90
	3×10^{-5}	6	92
	1×10^{-4}	24	—

Bayliss and Todrick suggested that Nu 1250 was insufficiently selective and that although 284C51 appeared to be slightly more efficient than 62C47 the difference might well be more apparent than

real. Little difference was observed between *iso*-OMPA and lysivane but the authors preferred the latter on account of the fact that with irreversible inhibitors the degree of inhibition varies markedly with the time of contact.

Two other selective inhibitors used in recent studies (Hobbiger, 1954, 1957) are 3-(diethoxyphosphinyloxy)-N-methylquinolinium methyl sulphate (3–0422, Roche) and 3-oxo-1 : 5-diphenylpentane-*pp'*-bis(propyl-dimethylammonium) dibromide (285C51, Wellcome). The former inhibits both cholinesterases at very low concentrations (10^{-8}M) while the latter is selective for AChE at a concentration of about 10^{-4}M.

Distribution of the Cholinesterases. Cholinesterases have been found in all vertebrates so far examined and in most of the invertebrates. AChE occurs as the predominant enzyme in nervous and muscular tissues and in erythrocytes. ChE is found in the blood serum, and in pancreas and salivary glands especially. There is, however, some degree of overlap between the two. Sympathetic ganglia have been reported to contain a mixture of AChE and ChE (Mendel and Rudney, 1944 ; Sawyer and Hollinshead, 1945), and cholinergic nerves have a higher activity than sensory ones (Umrath and Hellauer, 1948). In muscles AChE is present in high concentrations at the motor end plates, where it has been reported to be localized in the postsynaptic membrane (Couteaux, 1942, 1947). Erythrocyte AChE varies considerably in different species, being highest in man and in the guinea-pig. ChE has been reported in pancreas, ovary, Harderian glands, brown fat, liver, testis and parotid glands, *inter alia*. The esterases present in rat and guinea-pig liver are capable of splitting benzoylcholine although they will not hydrolyse acetylcholine (Blaschko, Chou and Wajda, 1947). They need not be characterised as ChE, however, since they split non-choline esters at a higher rate than benzoylcholine.

Methods for Non-specific Esterases (DW. 89)

Most of the substrates for the histochemical demonstration of esterases are hydrolysed by a number of different enzymes. For this reason it is difficult accurately to classify the methods which employ them. These are therefore set out in this chapter in sections which correspond to the enzyme or group of enzymes for which the method is *predominantly* employed. After consideration of the individual methods the histochemical use of inhibitors and activators will be discussed in a succeeding section. In applied esterase histochemistry insufficient attention has been paid to separation of the various types of enzyme by the use of inhibitors. It is absolutely necessary, in this as in other fields of enzyme histochemistry, to close the gap between this science and contemporary biochemical knowledge.

Simultaneous Coupling Azo Dye Methods

Naphthyl Acetates. A simultaneous coupling azo dye method for non-specific esterase was devised by Nachlas and Seligman (1949a) using β-naphthyl acetate as substrate. This ester and the α-naphthyl ester were equally rapidly hydrolysed by the tissues, but the authors expressed a preference for the former on account of the brilliance of the colours obtainable on coupling with diazonium salts. In this case they used a stabilized α-naphthylamine diazotate. Using cold acetone-fixed sections and avoiding contact with alcohol, incubation was carried out at pH 7·8 and at room temperature. A small amount of acetone (1 per cent. in the final solution) was needed in order to dissolve the substrate in the buffer solution. Incubation was continued for 20 minutes and, after washing in water, permanent mounts were made in an aqueous medium since the red azo dye was soluble in organic solvents.

Distribution of Enzyme. The distribution of esterase in the tissues of mammals, demonstrated by this method, was described by Nachlas and Seligman (1949b). In normal rat tissues esterase activity was found by these authors to be highest in lung (bronchial epithelium), liver, pancreas and kidney. These results agreed with those of Huggins and Moulton (1948) derived from tissue assay, using *p*-nitrophenyl acetate as substrate. Liver cells were uniformly stained with some accentuation in the region of the central vein.

Modifications of the Method. The original method of Nachlas and Seligman was found to be somewhat unsatisfactory. Even with the substitution of other diazonium salts (such as Fast Blue B salt) for the diazotate of α-naphthylamine, the final azo dye reaction product is not only soluble in alcohol and xylene but also in water. Gomori (1950a) first drew attention to this point, observing that part of the azo dyes produced by β-naphthol remained soluble for many minutes, *in vitro*, especially in the presence of acetone. The azo dyes produced by coupling with α-naphthol, on the contrary, precipitated instantly at pH 7. Gomori, therefore, suggested a modification of the technique using α-naphthyl acetate and the minimum of acetone necessary for its solution. He found by this method that several sites of esterase activity recorded by the β-naphthyl acetate method were uniformly negative. The advantages of using α-naphthyl instead of β-naphthyl acetate are partly illustrated by Figs. 140, 141, 142 and 143, in which the original method and the Gomori variation are compared. The dark purplish black colour developed with the latter is more easily visible than the red of the original method and localization within the cytoplasm of the renal tubular, and other, cells, is far more distinct. Using frozen sections, fixed in cold formalin for 10–16 hours, the results are

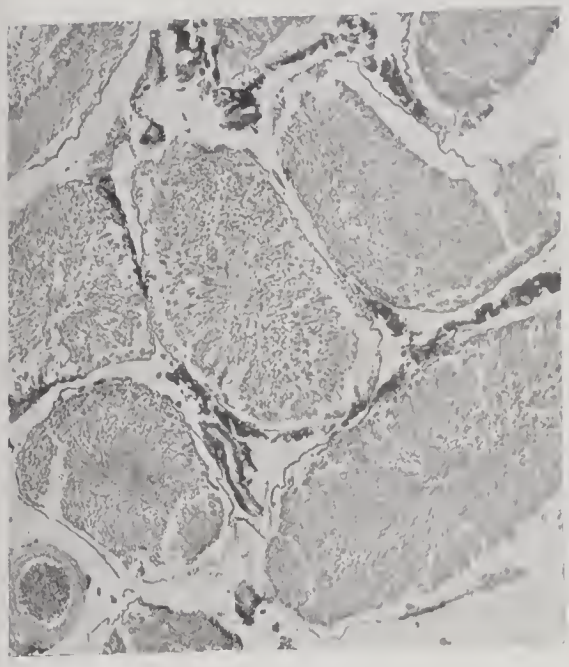


FIG. 140. Paraffin section (5·5 μ) of rat testis. Coupling azo dye method for non-specific esterase, using β-naphthyl acetate. Leydig cells and the adjacent tubular structures are stained. Diazotized *o*-dianisidine. × 60.

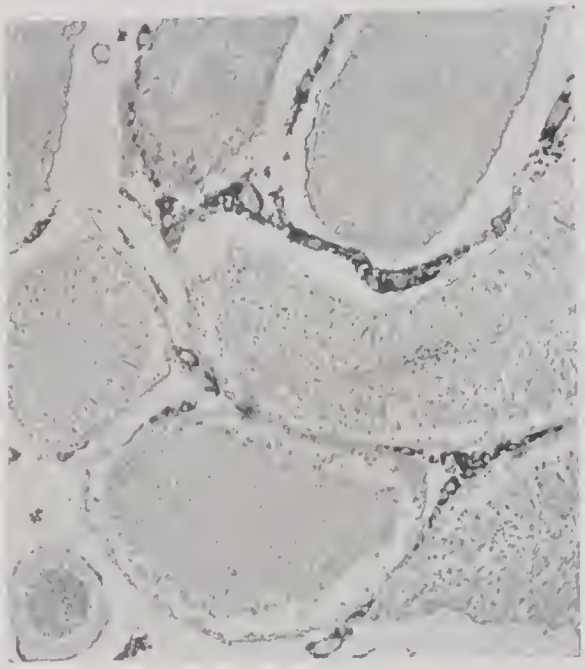


FIG. 141. As Fig. 140. Using α-naphthyl acetate. Only the cytoplasm of the Leydig cells contains dye particles. Diazotized *o*-dianisidine. × 60.

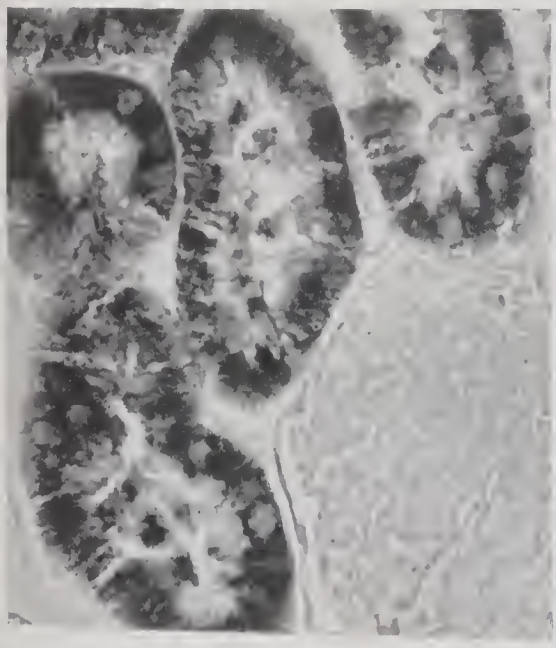


FIG. 142. Frozen section (10 μ) of rat kidney. Showing intracellular localization of esterase. α-naphthyl acetate method. × 400.

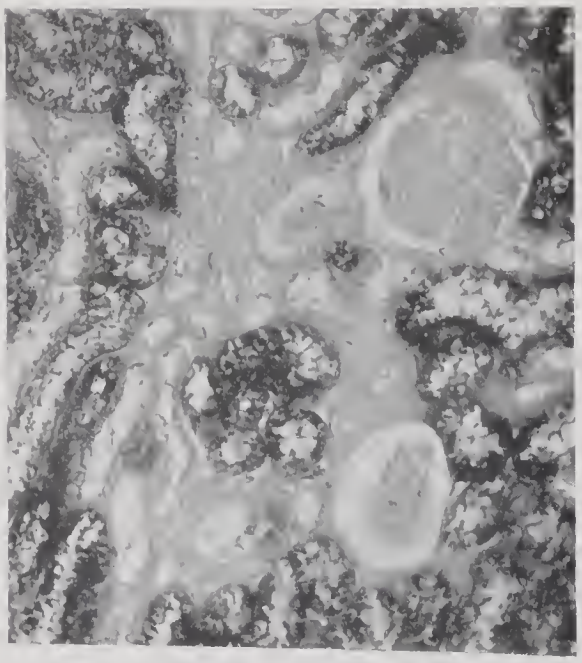


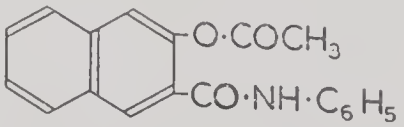
FIG. 143. Application of the β-naphthyl acetate method for esterase to rat kidney. Showing blurred outlines due to diffusion of the reaction product. Diazotized *o*-dianisidine. × 170.

[*To face p.* 464.

even better, since the distortion induced by acetone fixation is avoided and since about 80 per cent. instead of 30 per cent. of the enzyme remains active. When the β-naphthyl and α-naphthyl acetate methods are tested for diffusion by the method of face to face incubation of sections, one active, the other inactivated by treatment with heat and Lugol's iodine, diffusion of reaction product in the first ease exeeeds 150 μ. With the α-naphthyl aeetate method, using frozen sections, no diffusion of reaction product occurs at 75 μ, the lowest practicable spaee between the sections, and from examination of such sections it seems clear that any diffusion which occurs must be eonsiderably less than this. Fig. 143 illustrates the application of the β-naphthyl aeetate method to paraffin-embedded material. The red reaction produet is concentrated in the tubular epithelium but also diffused throughout the other structures in the section. The clarity of definition given by the α-naphthyl acetate method (Fig. 142 and Plate IVD), by eomparison, is excellent. For this reason the method has become one of the standard methods in applied histochemistry although it is doubtful whether the localization obtained can be considered aceurate at the cytological level.

Distribution of Enzyme by the Modified Method. Structures which were positive by the original method, but which were reported by Gomori to be negative with α-naphthyl acetate, were the parietal cells of the stomach (man, dog, rabbit), leueoeytes in the dog and the elastic fibres of large vessels in many animals. Gomori found that these sites could be stained by brief incubation with either α- or β-naphthol, followed by washing and coupling with a diazonium salt, suggesting that they were produced as artifacts and did not represent sites of esterase activity. The standard α-naphthyl acetate modifieation of the Nachlas-Seligman technique is given in Appendix 16, p. 886. This method is particularly suitable for applieation to pathological problems since it uses easily obtainable reagents and is easy to perform. Provided that certain precautions are taken, which are described in the Appendix, the results are absolutely reproducible.

Naphthol AS Acetates. Naphthol AS (2-hydroxy-3-naphthoic acid anilide) is used for the production of fast azo dyes in place of β-naphthol.



Naphthol AS acetate

Its acetate, which is easily prepared, was first used by Gomori (1952a and b) in place of α- and β-naphthyl acetates in the coupling azo dye method for esterases.

When coupled with a variety of stable diazotates Naphthol AS

produces colours resembling those given by β-naphthol. It is far less soluble in water than α- or β-naphthol, however, so that the Naphthol AS acetate methods possess the advantage that the initial reaction product (Naphthol AS) is less likely than the other two naphthols to diffuse from its site of production before coupling occurs.

Differences in the localization of esterase in cold acetone-fixed sections by the α-naphthol and Naphthol AS variants led Gomori (1952b) to postulate the existence of a specific "AS esterase." The differences in frozen sections, however, appeared to me to be much smaller and I therefore stated (1954) that they were insufficient basis for the supposition that a separate enzyme was involved. This view has to be modified in the light of further investigations which are reported below.

Using Naphthol AS acetate, with a variety of stable diazotates, in a standard method for esterase (Appendix 16), I found the final product to be particulate in every case. Plate VA and Fig. 144 show the results obtained, with guinea-pig kidney fixed in cold formalin and cut on the freezing microtome, using Naphthol AS acetate and the stable diazotates of 4-chloro-*o*-anisidine and 4-benzoylamino-2 : 5-dimethoxyaniline respectively. They should be compared with Plate IVD and Figs. 141 and 142. Considerable improvement in the precision of esterase localization was produced by the use of substituted AS-naphthol acetates (Pearse, 1954). Three substrates of this type were prepared from Brenthols* AS-BG, AS-OT and AS-FR. These were, respectively, 2-acetoxy-3-naphthoic-2,5′-dimethoxyanilide, 2-acetoxy-3-naphthoic-*o*-toluidide and 2-acetoxy-3-naphthoic-*o*-anisidide. In Germany and the United States the equivalents of these last two substrates are AS-D acetate and AS-OL acetate. With the first, the final reaction product (FRP) was invariably macrocrystalline, but with the other two the results, especially in free-floating frozen sections, were much better than those obtained with Naphthol AS-acetate.

Further tests with acetates of substituted AS-naphthols were carried out by Burstone (1957a and b) and by Goessner (1958). Both these authors obtained excellent results with AS-D acetate. Goessner used his substrates in 0·1M-phosphate buffer at pH 6·8 with Fast blue BB (Plate VD) or Fast Garnet GBC salt (18). Burstone (1957a) used AS-D acetate in 0·2M-tris buffer at pH 7·1 with Fast red RC (7) or Fast Garnet salts. Later (1957b) he employed AS-LC acetate at the same pH with Fast red-violet LB salt. Burstone observed with this last substrate that the distribution of activity was essentially the same as with other naphtholic substrates but that sharper localizations were obtained. In the case of freeze-dried sections of developing bone, however, esterolytic activity was observed in the bone matrix which

* I.C.I. Ltd. equivalents of AS-Naphthols.

could not be demonstrated with the usual naphtholic substrates. The activity of this enzyme was resistant to E-600 and to DFP but it was inhibited to some extent by Cu^{2+}, Cd^{2+} and Zn^{2+} ions and also by sodium fluoride. It thus falls into the category of an A-esterase, but further studies may well show that it belongs to the C-esterases and functions as a cathepsin (Bergmann *et al.*, 1957) (see Chapter XXI, p. 591).

The question which confronts us with the Naphthol AS acetates is whether these should be employed in place of the simple naphthol esters in routine histochemical investigations or whether they should be reserved for more specialized studies. I consider that for surveying the distribution of all surviving types of non-specific esterase in paraffin-embedded tissues the substituted AS acetate methods are excellent. On the whole, I prefer AS-D acetate to AS-LC acetate. With formalin-fixed frozen sections, especially if these are mounted on slides before incubation, the results are less happy. Nevertheless, even in preparations such as these a sharper definition of FRP is obtained with AS-D acetate and Fast blue BB salt or Fast black K salt (14). This is not necessarily the most accurate localization of enzyme, however, and probably the α-naphthol acetate method will continue in general use for the time being.

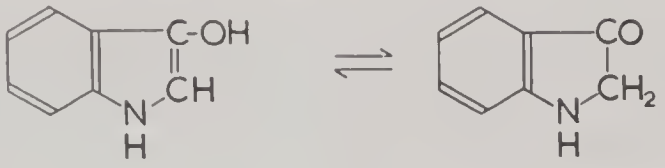
Other Substituted Naphthol Acetates. The only other substrate in this class which has been employed in a simultaneous coupling method for esterases is 6-bromo-2-naphthyl acetate. This was used by Burstone (1957a), who found that it was not hydrolysed by the bone matrix enzyme. In frozen sections the results with this substrate are comparable with those obtained with simple naphthol esters. It has no real advantage over the latter although theoretical considerations (Chapter XIII, p. 278) would lead one to expect that it might have.

Methods Depending on the Production of Indigo

Histochemical methods based on the use of an entirely new principle were described, independently, by Barrnett and Seligman (1951) and by Holt (1952). In both cases the methods evolved have initially been applied to the demonstration and localization of esterases and for this reason the whole principle of enzyme demonstration by means of indoxyl substrates is considered at this point. With the synthesis of new indoxyl substrates, satisfactory methods seem likely to be produced not only for esterases but also for phosphatases, sulphatases, glucosidases and glucuronidases. Extension to the peptidases is also possible. The acetates of indoxyl esters have been employed by Holt in a long series of studies culminating in the production of an esterase substrate (5-bromo-4-chloroindoxyl acetate) capable of very precise localization of enzyme activity. Reviews of these studies have been

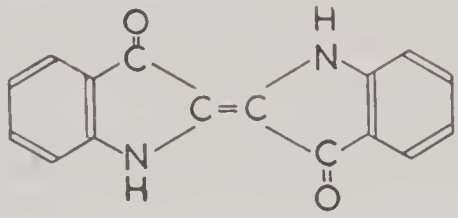
given by Holt (1956, 1958), and these should be consulted by those who intend to work in this promising field of enzyme histochemistry.

Principles of the Indoxyl Methods. In all these methods the principle involved is the hydrolysis of a soluble ester of indoxyl, with the liberation of the free indoxyl. This normally exists as a tautomeric mixture of substances I and II.



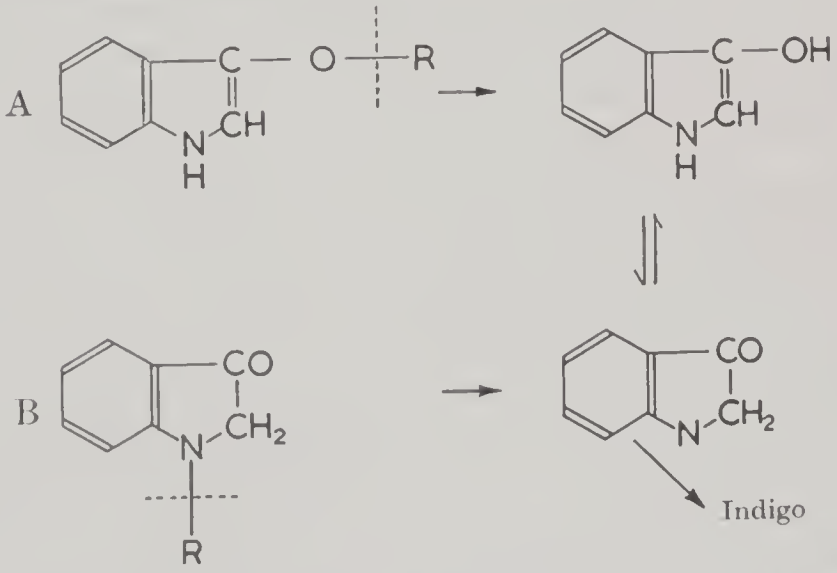
I II

The equilibrium normally favours the production of II, which is rapidly oxidized by atmospheric oxygen to the completely insoluble bright blue indigo III.

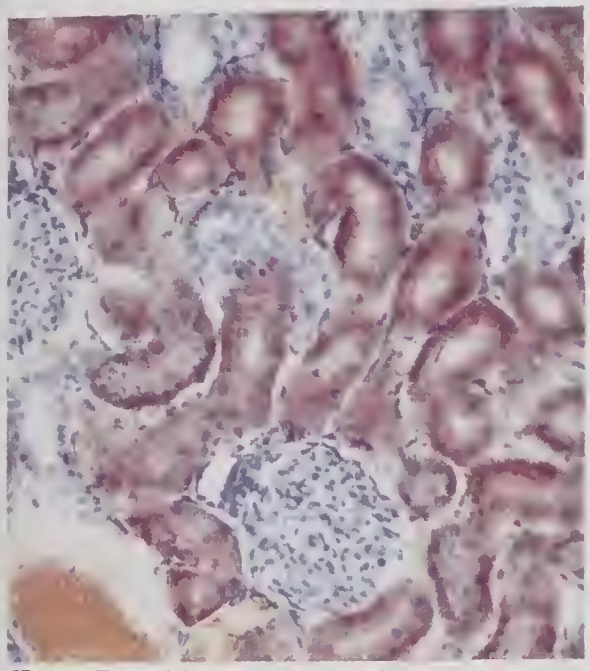


III

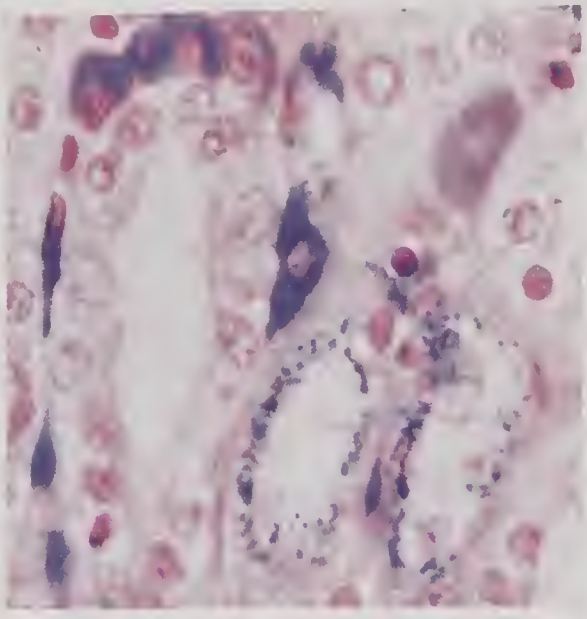
The demonstrating agent is thus the simplest possible, molecular oxygen, and the formation of indigo in the presence of air will be induced by hydrolytic enzymes catalysing either of the two processes, A and B, for which the equations are given below.



As will be observed from the above equations, indoxyl has two main reactive centres, one in the enol-hydroxy group (A) and the other in the imino- group (B). From the point of view of histochemistry the most important indoxyls are those derivatives which are substituted on the enol-group.

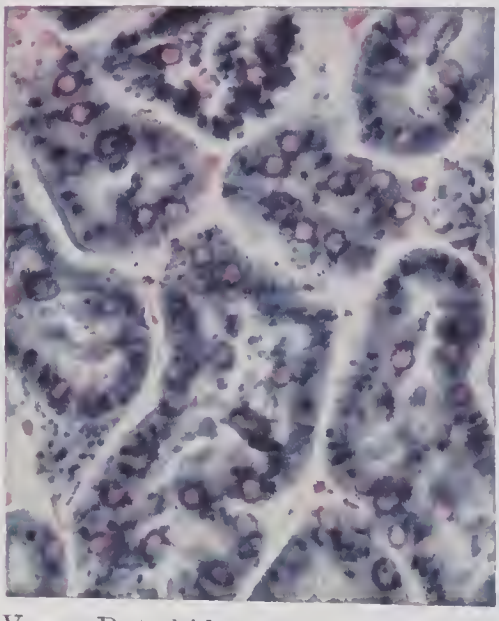


VA. Rat kidney (10 μ frozen section). AS-esterase in the convoluted tubules. Incubation 30 minutes. Coupling azo dye method, Mayer's haemalum. × 130.



VB. Rat kidney. Twenty days' potassium depletion. Large interstitial cells containing a strong esterase appear between the tubules. 5-Bromoindoxyl acetate—Carmalum. × 335.

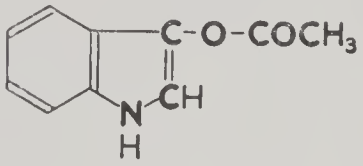
VC. *Buccinum undatum* (whelk). Oocyte. Non-specific esterase restricted to a few circumscribed zones, possibly centrosomes. 5-Bromoindoxyl acetate—Neutral red. × 460.



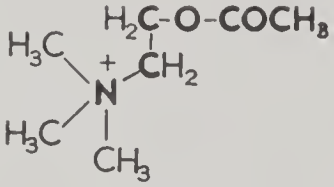
VD. Rat kidney. Intracellular localization of non-specific esterase. Naphthol AS-D acetate—Fast blue BB, Feulgen. × 100.

[*To face p.* 468.

Application of the Indoxyl Methods. Two substrates were used by Barrnett and Seligman (1951) for histochemical application, (1) indoxyl acetate (*O*-acetylindoxyl) and (2) indoxyl butyrate (*O*-butyrylindoxyl). Holt (1952) used these two compounds and later (Holt and Sadler, 1958a) some forty other mono-, di- and tri- substituted indoxyls. He drew particular attention to the structural resemblance of *O*-acetylindoxyl to acetylcholine,



O-acetylindoxyl



Acetylcholine

and he showed, *in vitro*, that the former was rapidly hydrolysed by specific cholinesterase. When the enol-group was substituted by larger acyl groups (to some extent even with *O*-butyrylindoxyl) or by other types of grouping altogether, hydrolysis by cholinesterases became negligible. Esterases and lipases, however, continued to hydrolyse the molecule with the consequent production of indigo.

With 0·0004 M-*O*-acetylindoxyl in 0·1 M-veronal acetate buffer (pH 8·5) as substrate, Barrnett and Seligman used an incubation period of 10 minutes with fresh frozen sections. With *O*-butyrylindoxyl the incubation time was 15–20 minutes. A blue precipitate of indigo was produced in the majority of tissues in these times and the form of the precipitate varied between numerous small particles, in kidney for instance, to a few large star-shaped crystals in single fat cells. Seligman, who apparently hoped that the indigo would be deposited in submicroscopic particles, observed that the cause of the large size of the granules was not altogether clear. Holt (1952) also remarked that the reasons for the precipitation of the dye in large granules or crystals were not clear. Certainly localization of esterases with the two substrates employed by Barrnett and Seligman was extremely poor (see Fig. 145).

Improvement was to be expected from three sources ; first, by the addition to the substrate solution of substances altering the character of the precipitate ; secondly, by the provision of an efficient capture reaction (see Chapter XIII, p. 279), and thirdly, by modification of the substrate molecule at points other than those which are the sites of enzyme attack.

The growth of crystals is favoured by an acid pH and by a high lipid content of the tissues. Excessive solvent (ethanol) for the indoxyl substrate also favours this type of crystallization. The process is diminished by using an alkaline pH, and only above pH 9·0 are the

precipitates from unsubstituted indoxyls of reasonable size. It was observed by Holt and Withers (1952) that indoxyl itself, and halogen-substituted indoxyls, liberated at sites of high enzyme activity, could diffuse widely before complete oxidation to insoluble indigoid dyes could take place. They found that this process was greatly reduced by traces of Cu^{2+} ions in the incubating medium and that this effect was due to the catalytic action of the metal on oxidation of the liberated indoxyl. A series of oxidation catalysts was tested and potassium ferricyanide was found to be the most satisfactory in practice. A tendency for further oxidation to colourless dehydroindigo was prevented by addition of an equimolar solution of potassium ferrocyanide. The kinetics of aerial oxidation of indoxyl and some of its halogen derivatives were the subject of a special study by Cotson and Holt (1958) who found that, in the presence of excess O_2, oxidation was first order with respect to indoxyl under alkaline conditions. Under acid conditions, however, the reaction was much slower. In the absence of a catalyst the oxidation rates were found in all cases to be inadequate for accurate histochemical localization of enzyme. Holt and Withers (1958) reported the effect of changes in oxidation rate on the staining pattern and showed, for instance, that diffuse staining in many types of tissue was reduced to discrete intracellular localization of indigo deposits when the concentration of oxidation catalyst was sufficiently high.

Holt, in several of his papers, has maintained that the standard, oxidation catalyst has little effect on esterases. The fact remains however, that the incubation period necessary to produce comparable amounts of indigo from identical tissues is very greatly increased by ferricyanide in the concentration employed. It is at least possible that some of the more precise localization is due to differential inhibition of esterases, or to increased loss of lyo-esterase by diffusion into the incubation medium during the prolonged incubation period.

Staining of Red Cells. In the first edition of this book the view was expressed that staining of red blood cells, which takes place when indoxyl acetates are used in the absence of oxidation catalysts, was due to the latter preventing the substrate from gaining access to the contained red cell esterase. This view was derived from Holt and Withers (1952). The subsequent studies of Cotson and Holt (1958) have shown that aerial oxidation of indoxyls results in the production of hydrogen peroxide and the current view of the mechanism of red cell staining is that a peroxidase-like compound within the red cell (? hæmoglobin) oxidises the diffusing indoxyl to indigo. Considerable reduction of the red cell reaction can be obtained by addition of 10^{-2} M-azide, cyanide, sulphide or hydroxylamine, and also by excess H_2O_2. The effect of these peroxidase inhibitors suggests that the above explanation

is correct. The action of ferricyanide on the red cell reaction may be by inhibition of the peroxidase effect or, possibly, by prevention of diffusion of the indoxyl into the red cell.

Improvements of an entirely different order have been brought about by the use of mono- or dihalogen-substituted indoxyl acetates as substrates. Holt and Withers (1952) used the first of a long series of halogen-substituted indoxyl acetates, 5-bromoindoxyl acetate, which gave rise to extremely small crystals of 5-5′-dibromoindigo. This is one of the more insoluble indigoids, but it has considerable solubility in lipids and, where these are present in large amounts, crystallization can still occur. Elsewhere the microcrystals are small enough not to be distinguished except under the higher powers of the microscope (see Plates Vb and Vc and Fig. 146). Further studies on the desirable properties of indigogenic substrates (Holt and Sadler, 1958b) have led to the selection of 5-bromo-4-chloroindoxyl acetate as the optimal reagent for the histochemical demonstration of esterases. This gives rise to a highly substantive indigoid dye whose deposits in the tissues are not crystalline. They are, however, less strongly blue than those of some of the other halogen-substituted indigos. Although 5,5′-dibrom-4,4′-dichloroindigo is lipid soluble it does not form large crystals in fat cells as does 5,5′-dibromindigo. This effect is considered to be due to its substantivity for protein.

Results of Indoxyl Methods. Indoxyl acetates are hydrolysed by carboxylic esterases of many different types (Underhay *et al.*, 1956 ; Hobbiger, 1957). They can therefore be used for the histochemical demonstration of any or all of these esterases in a variety of types of preparation. The best results are obtained with formol-calcium-fixed frozen sections which can be processed by the special procedures of Holt and Withers (1958). These involve impregnation of the block, after fixation, in a gum-sucrose solution at 0–2° for 24 hours before cutting sections. The latter are preferably used as free-floating sections rather than mounted on slides. Besides the substrate and sufficient ethanol to carry it into solution, the incubating medium (Holt, 1958) contains the oxidation catalyst, 1·0 M-NaCl, and 0·01 M-$CaCl_2$. The sodium chloride is presumed to act by reducing diffusion of the lyo-esterase into the incubating medium and it might well be replaced by PVP or methyl cellulose. The precise function of the calcium ions is not clear. Their omission causes blurring of the sharp localization usually obtained and Kambli (1958) has suggested that they may act by forming an insoluble calcium-indoxyl complex which is subsequently oxidized to indigo.

As well as the blue indigo reaction product most workers with indoxyl substrates have observed a red reaction product, more marked in some tissues than in others. This product is especially noticeable in

brain tissues where it stains the white matter a delicate pink colour, increasing in intensity on storage in glycerine jelly. Cotson and Holt (1958) have shown that during the formation of indigoid dyes from indoxyls small amounts of isatins are formed. It is these which are apparently responsible for the pink and red colours observed in sections incubated with indoxyl substrates.

Formation of Indigo as a Function of pH. An important point was made by Holt (1958) which re-emphasizes his earlier views on the mechanism and rate of production of indigo. When ferricyanide is present in excess the oxidation of indoxyls proceeds in two directions, one towards indigo and the other towards colourless dehydroindigos. As the reaction medium becomes more alkaline the amount of indigo decreases and the amount of dehydroindigo increases. At acid pH levels the converse is true and the amount of indigo deposited is greater than at alkaline levels. According to Holt the effect of changes of pH on indigo production is much greater than their effect on esterase activity and he has stated that Pearson and Defendi (1957) were thus led erroneously to conclude that the pH optimum for indoxyl esterase was 4·7. In rat brain, Pepler and Pearse (1957a) found that indigo production after hydrolysis of 5-bromoacetylindoxyl was maximal at pH 6·2, irrespective of the type of esterase responsible. However, they also found that a sharp fall in indigo production, with four of the six esterases, was produced by fractional lowering of the pH values. These observations support Holt's views to some extent but suggest that he has underrated the importance of enzyme activity.

Although it might appear that the effects recorded above constitute a serious indictment of the indoxyl methods, Holt declares that, since evidence for a first order rate is available for the production of indigos from indoxyls, the dye must everywhere be formed in proportion to the amount of indoxyl liberated by enzyme action. It would seem, nevertheless, that if the ratio of indigo to dehydroindigo varies with the pH absolute quantitation of enzyme activity will be difficult unless a correction can be simply applied. This will not concern the majority of histochemists, but it must be realized that comparisons can only be made between one tissue and another if the pH remains constant.

Methods for Lipases (DW. 90)

The "Tween" Methods

Gomori (1945) observed that lipase was not destroyed by acetone fixation. According to this author it had apparently a small and moderately diffusible molecule so that it was essential to fix tissues as soon as possible after removal if accurate histochemical localization was desired. After fixation and dehydration in acetone lipase was

found to be moderately resistant to the action of heat, and the paraffin embedding technique was therefore employed. The method evolved by Gomori, which underwent many modifications in the hands of its original author, depends on the incubation of acetone-fixed paraffin sections in the presence of water-soluble esters of long-chained saturated fatty acids with polyglycols or polymannitols, in the presence of calcium ions. These substances are commercial detergents and the name "Tween," which is attached to the series most often used, can reasonably be attached to the method as a whole, although other substrates besides those belonging to the Tweens can be employed. On hydrolysis the liberated fatty acids form insoluble calcium soaps which are deposited at the sites of enzyme activity ; subsequently these are converted into lead soaps and thence to the brown sulphide of lead. The duration of incubation varied from 12–48 hours, depending on the tissue employed, and control sections were provided by slides inactivated either by short heating (boiling water, 10 minutes), Lugol's iodine (1 minute), or 5 per cent. phenol (10 minutes). In the original method glycerol was used as an activator and maleate buffer at pH 7·4. Later, glycerol was found to have no activating effect under the conditions of incubation and, since the buffering power of maleate was found to be inadequate, a tris(hydroxymethyl)aminomethane, $(CH_2OH)_3CNH_2$, buffer (pH 7·2 to 7·4) was used in its place (Gomori, 1946, 1949, 1950a). The final method (Gomori, 1952b) is given in Appendix 16. Other authors have published modifications of the original lipase technique including Wachstein (1946), Stowell and Lee (1950), and Mark (1950). The latter observed that acetone fixation destroyed a large amount of lipase and he therefore used formalin-fixed frozen sections instead of the more usual acetone-fixed ones. His modification of Gomori's method is also given in Appendix 16. Sneath (1950) made the comment that the lipase method of Gomori had been little used in morbid histology. As the result of a critical study of the original method, and Wachstein's modification, he concluded that both methods required further improvement before they would be reliable for diagnostic work. Richterich (1951), however, claimed satisfactory results in various tissues which his illustrations did little to substantiate. The same technique was used by Buño and Marino (1952), with Tween 60 as substrate, in a study of the "lipases" of the developing chick embryo. These authors stated that they were unable to distinguish lipase from non-specific esterase activity. Later studies by Martin (1953b), using Tween 40, revealed strong "lipase" activity in many organs in a variety of mammalian species, in addition to pancreas and liver. Especially strong activity was found in the ducts of the salivary glands in all species except the rat. No inhibitors or activators were used in these studies and therefore no distinction

between true lipases and non-specific esterases could be made. A more recent study by Sinrala (1956) of the "lipase" activity of human and monkey gastric mucosa employed Tweens 20, 40, 60 and 80. Again no attempt was made to distinguish lipase from esterase activity.

The variability of the results obtained by the lipase method is due to several factors. Postmortem autolysis, as already suggested, causes diffusion of enzyme in the tissues while prolonged fixation diminishes the amount of enzyme available. A similar result is effected by excessive or prolonged heating during embedding or mounting on the slides (no more than 58° for 3 hours should be permitted for the former or than 37° for the latter). Floating sections on water instead of acetone may cause some loss of enzyme or disturbance of distribution, and storage in the paraffin block also causes a gradual diminution in activity. The maintenance of the pH in the region of 7·4 during incubation appears to be one of the main difficulties of the method, though Gomori's "tris" buffer is very efficient in the physiological range. Another factor which may influence the result is the presence of varying amounts of free fatty acid in different batches of the same substrate. If large amounts of these are present the incubating mixture gradually becomes turbid, due to the formation of calcium soaps which settle on the slides and may be indistinguishable from the products of enzymic activity in the tissues. Gomori recommended removal of free fatty acids by incubating the turbid mixture at 37° for a few days and filtering through glass wool. Bacterial decomposition of the substrate was avoided by the use of a preservative such as thymol or chloretone. Merthiolate, which was originally used, was found to be a weak inhibitor of lipase. Modifications introduced by Martin (1953a) certainly produced cleaner and more reproducible results. He employed the technique of Ruyter and Neumann (1949), using free-floating, un-dewaxed paraffin sections and mounted these in glycerine jelly after rapid removal of wax with trichlorocthylene. This manœuvre followed the recommendation by Gomori (1951) that xylene should be avoided as it caused rapid fading of the final product.

Specificity of the Tween Method. Gomori (1950b) remarked that the topography of enzymic activity by the Tween method was not identical with that shown by the Nachlas-Seligman method for esterase. Certain cells of the cardiac portion of the mouse stomach and cellular debris in the tubules of the testis, were intensely Tween-positive but esterase-negative. On the other hand, the superficial cells of the gastric mucosa and the interstitial cells of the testis (man, rat, rabbit) stained much more intensely by the azo dye method. As the result of some studies on the histochemical specificity of his method, in which thirty-five different water-soluble long-chained (C_{12}

to C_{18}) fatty acid esters were employed, Gomori (1949) reported that the true lipases could hydrolyse esters of unsaturated fatty acids rapidly, a property not possessed by the non-specific esterases. This difference, reinforced by the difference in response to small concentrations of taurocholate, could serve to distinguish the two enzymes, but little use seems to have been made of it in practice.

Application of the Tween Method. For routine application to pathological problems, or even for normal tissues, the Tween method cannot be recommended. Except in the pancreas, where uniform results are consistently obtainable, I found that the final precipitates of lead sulphide are always patchily distributed. Fig. 149 shows the kind of result obtained by application of the method to formalin-fixed frozen sections, in this case a section of rat liver, along the lines of the modification introduced by Mark (1950). In the first edition of this book (1953) I stated that as a starting point for further research on the Tween methods a standard 16-hour fixation in 6 per cent. neutral formalin at 4° should be used, and that much further work would be necessary before the theoretical advantages of using substrates resembling the natural substrates for the lipases could be translated successfully to routine histochemistry. I cannot see any reason, at the present time, to modify this view.

Methods for Cholinesterases (DW. 96 and 97)

Methods using Higher Fatty Acid Esters of Choline

Gomori (1948) produced a method for demonstrating cholinesterase in the tissues, resembling his method for lipases, in which a variety of higher fatty acid esters of choline were used as substrates. The four alternative substrates which he evolved were lauroyl, myristoyl, palmitoyl and stearoyl choline, and the second was found to be the most satisfactory for histochemical purposes. Thin slices of tissue were fixed in cold acetone and double embedded in celloidin and paraffin. Sections from these blocks were incubated for 2–16 hours at 37° in 0·1 M-tris(hydroxymethyl)aminomethane buffer, at pH 7·5 to 7·8, in the presence of the substrate (0·0004 M-myristoyl choline), cobalt ions, and traces of $CaCl_2$, $MgCl_2$ and $MnCl_2$. On hydrolysis the free fatty acid combined with the cobalt to give a fine granular white precipitate, easily rendered visible by subsequent treatment with yellow ammonium sulphide. Calcium, strontium, barium and magnesium were originally tried in place of cobalt but all gave a coarse crystalline precipitate unsuitable for precise localization. Ca^{++}, Mg^{++} and Mn^{++} ions were found to activate cholinesterases *in vitro* (Nachmansohn, 1940) and they were employed by Gomori for this reason. Higher concentrations of substrate than 0·001 M inhibited the enzymes

and the particular buffer was chosen because it was found to cause minimal inhibition.

Theoretical Considerations. Gomori showed that crude extracts of various animal tissues would hydrolyse the higher choline esters at varying rates, but notably poor hydrolysis was produced by rat brain homogenates. Brain tissue was stated by Mendel and Rudney (1943) to contain only AChE, and Gomori's finding, therefore, suggested that the higher choline esters were not attacked with any ease by AChE. In no case did the rate of hydrolysis of the artificial substrates by nervous tissue approach that for acetylcholine. Purified bovine erythrocyte AChE, and a purified AChE from the electric organ of *Torpedo*, failed to hydrolyse the myristoyl, palmitoyl or stearoyl esters although the first two did hydrolyse the lauroyl ester. *Torpedo* AChE, probably the purest of the three, would not hydrolyse lauroyl choline. In view of these *in vitro* results Koelle and Friedenwald (1949) concluded that the myristoyl choline method localized "only non-specific esterases," meaning non-specific cholinesterases, but in spite of Gomori's *in vitro* results, Denz (1954) found that AChE (red cell) hydrolysed myristoyl choline at a slow rate. This was apparently sufficient to produce a positive histochemical result, even with short incubation, since in Denz' fresh frozen sections the enzyme was fully active and the sensitivity of the method was therefore high.

Distribution of Enzyme. The distribution of enzyme shown by the myristoyl choline method differs to some extent from that shown by a non-specific esterase method such as that of Nachlas and Seligman, or its α-naphthyl acetate modification. In particular, tissues such as brain and spinal cord, in which various components react by the Gomori method, have been reported as negative by the Nachlas-Seligman technique. Some interesting locations are demonstrated by the former, including ganglion cells, structures in striated muscle identified as muscle spindles, and the smooth muscle of the bladder, uterus and gastro-intestinal tract in several species ; Gomori, however, did not give a full list of localizations in his paper. Dog and mouse tissues gave most intense pictures with the lauroyl and myristoyl esters and human tissues with palmitoyl choline. Rat tissues seem to be unable to hydrolyse myristoyl choline at a sufficiently rapid rate to give a positive histochemical result.

Hard and Peterson (1950) used the myristoyl choline method to study the distribution of cholinesterase in nerve tissues of the dog. They obtained largely consistent results and attributed occasional erratic or incomplete reactions to variations in concentration and pH of the substrate solution. In the central nervous system the reaction was confined almost entirely to nerve fibres, in nerve cells the reaction was slight but the nuclei and nucleoli often stained. In

FIG. 144. As Fig. 142. Localization of esterase by a naphthol AS acetate method, showing large steely blue dye particles deposited mainly in the primary convoluted tubules. Diazotized 4-benzoylamino-2 : 5-dimethoxyaniline. × 400.

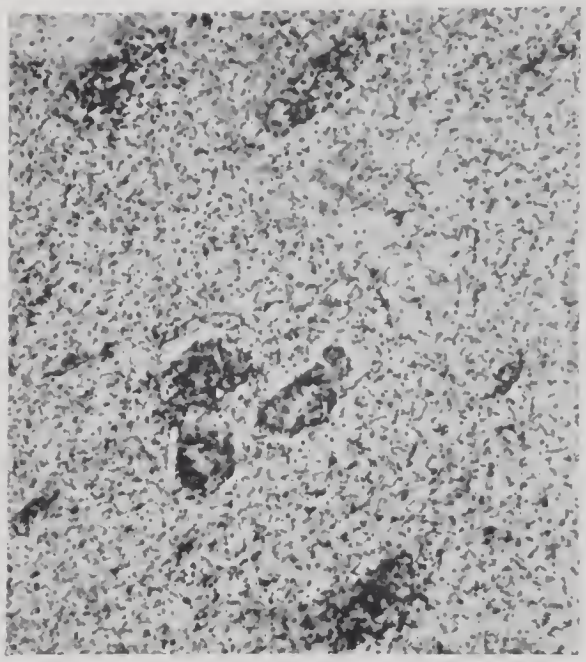


FIG. 145. Frozen section (10 μ) of rat spinal cord. Showing crystalline precipitates of indigo in the anterior horn cells and elsewhere. Indoxyl method for cholinesterase (substrate *O*-acetylindoxyl). × 270.

FIG. 146. Formalin-fixed frozen section (10 μ) of rat diaphragm. A number of motor end plates can be seen and structural detail is excellent. (Incubation 10 minutes.) *O*-acetyl-5-bromoindoxyl method. × 160.

FIG. 147. Rat diaphragm. Motor end plate. Shows concentration of specific acetylcholinesterase in the subneural apparatus. 4-Chloro-5-bromoindoxyl acetate. × 2,000.

[*To face p.* 476.

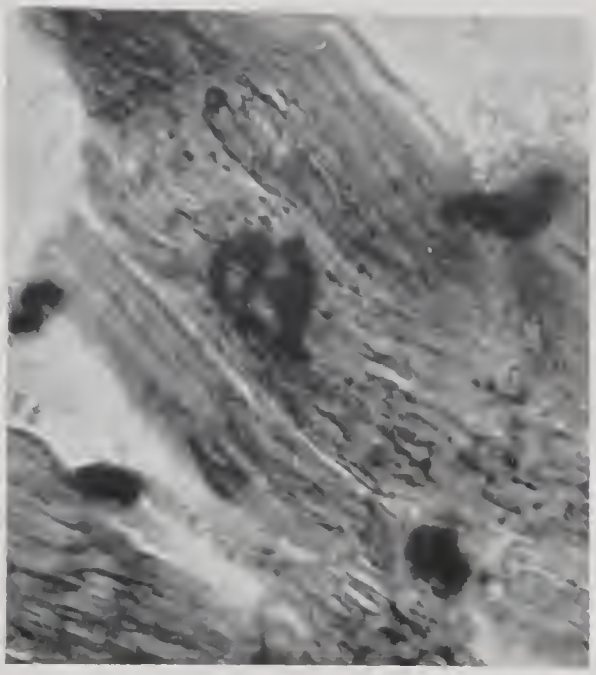


FIG. 148. Rat diaphragm, unfixed frozen section (20 μ). Showing a group of five motor end plates. Some structural detail is visible in the central one. (Incubation 30 seconds.) α-naphthyl acetate method. × 430.

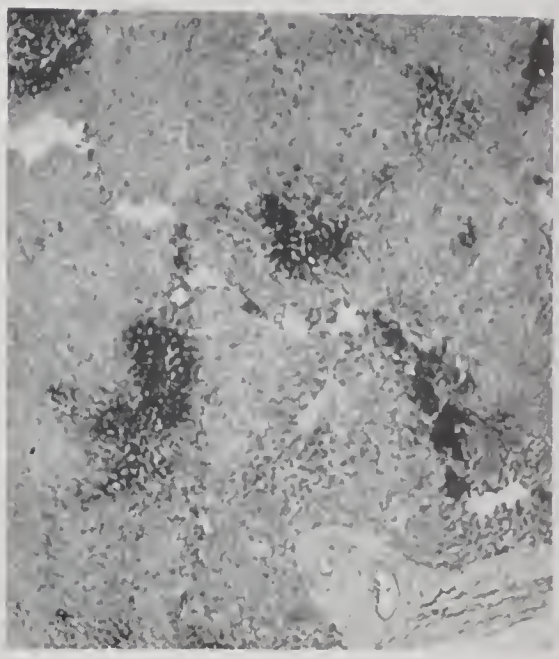


FIG. 149. Formalin-fixed frozen section (10 μ) of rat liver. Tween method for lipase (substrate Tween 60), showing patchy localization. × 24.

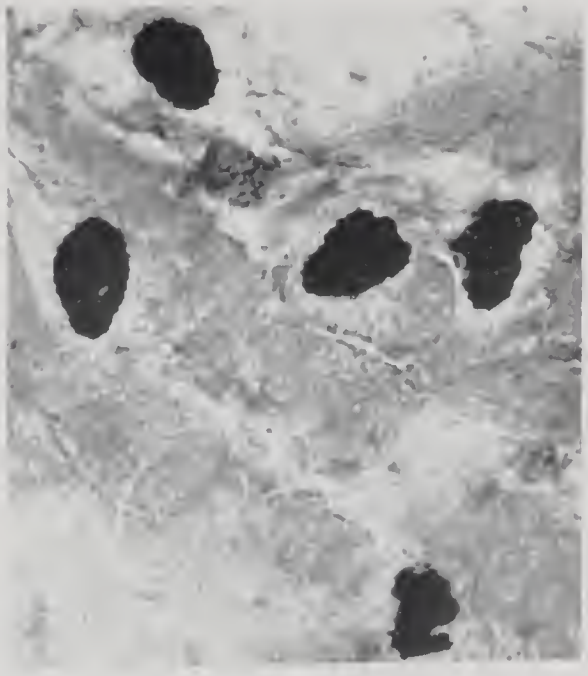


FIG. 150. Rat diaphragm, unfixed frozen section (20 μ). Showing a group of motor end plates. (Incubation 60 minutes.) Myristoyl choline method. × 430.

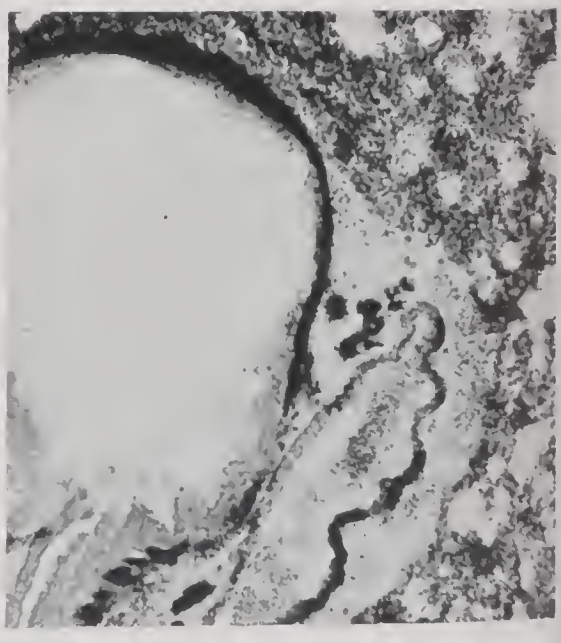


FIG. 151. Mouse lung, acetone-fixed paraffin section (5·5 μ). Showing, at upper left, the medial coat of an artery and, below, bronchial epithelium. (Incubation 16 hours.) Myristoyl choline method. × 20.

the peripheral nervous system a strong reaction was localized at the margins of both spinal and sympathetic ganglia. Since the enzyme concerned was inhibited both by 10^{-5} M-prostigmine and by excess of substrate, the authors were inclined to regard it as a specific cholinesterase. In a series of further studies with myristoyl choline as substrate, Hard, Peterson and Fox (1951) obtained evidence that the abolition of the histochemical reaction in sympathetic ganglia after transection of the cord, was closely related to the fall in AChE measured in the same tissues by biochemical means. These findings were regarded as further evidence that the enzyme demonstrated was AChE. Nevertheless, none of the evidence offered precluded ChE being responsible for their reactions.

Application of the Method. Denz (1953) found that the myristoyl choline method was exceedingly capricious with acetone-fixed tissues, sections from the majority of blocks giving negative results. He used instead fresh frozen sections and with these the method was absolutely reliable and reproducible. With fresh tissues the incubation time is reduced from an average of 8 hours to less than 30 minutes ; localization is sharp and diffusion artifacts are not in evidence. Fig. 150 shows motor end plates in rat diaphragm stained by the myristoyl choline technique, and Fig. 151 illustrates the application of the method to an acetone-fixed section of mouse lung. Staining is most intense in the arterial walls (upper left) and much less in the bronchial mucosa (lower right).

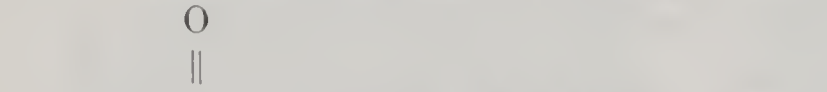
The Gomori technique for cholinesterases, if used with fresh or suitably fixed material, is thus seen to be capable of demonstrating both AChE and ChE. With the former, the rate of hydrolysis is far slower than that obtainable with other histochemical substrates, but with ChE the rate of hydrolysis is excellent. If a positive result is obtained with the myristoyl choline method in formalin-fixed frozen sections, using short incubation (Appendix 16), the participation of AChE is largely excluded. It is still necessary to distinguish between ChE and non-specific esterases and this can be done by using specific inhibitors for ChE in suitable concentrations.

The Gomori method has not been found useful for routine investigations but it may still be found valuable as a control method for other methods for cholinesterases described below, especially where ChE is concerned. The only extensive histochemical study using higher fatty acid esters of choline as substrates is that of Carbonell (1956). This author demonstrated the conducting system of the heart in several mammalian species, including man, with palmitoyl-, myristoyl-, and pentadecanoyl-choline. The extent and distribution of the conducting system of the heart is difficult to study by any method other than one capable of showing cholinesterase activity. Carbonell found that the

best results were obtained with pentadecanoyl-choline. Activators ($MgCl_2$, $MnCl_2$ and $CaCl_2$) employed in the original method were not found useful and the incubation period was 5–6 hours.

Methods Using Acetyl- or Butyrylthiocholine

The Original Method. Koelle and Friedenwald (1949) employed a substrate (acetylthiocholine) which is hydrolysed by AChE and ChE at a more rapid rate than acetylcholine itself. It was presumed by these authors that the increased rate of hydrolysis was due to the



—C—S— linkage being weaker than the —C—O— linkage of acetylcholine. The original histochemical procedure consisted of incubating fresh frozen sections (or teased preparations) in a medium containing 0·004 M-acetylthiocholine or butyrylthiocholine, 0·002 M-copper sulphate and 0·008 M-glycine at pH 8·06 for 10–60 minutes. On hydrolysis the liberated thiocholine was presumed to react with the copper salt to give a relatively insoluble product, copper thiocholine. In order to facilitate precipitation, and to prevent diffusion, the incubating medium was saturated with copper thiocholine before the sections were placed in it. This was a critical step in the performance of the original method and it remained of importance in subsequent modifications of the method introduced by Koelle (1950, 1951). Unless complete saturation with copper thiocholine was achieved subsequent localization of the enzyme was poor and diffusion evident. Much sharper localization of the precipitate was obtained by controlling the pH. It was considered that no false reactions were caused by initial saturation with the reaction product since it was observed that inactivated control sections, incubated in the same solution, showed no staining. These controls were prepared by allowing freshly-cut sections to stand in 0·001 M-di-*iso*propylfluorophosphate (DFP)

in 0·85 per cent. saline, for 30 minutes at room temperature. (At this concentration DFP irreversibly inhibits both AChE and ChE). After thorough washing, the control sections were transferred to the incubating medium and, after incubation, both test and control slides

were washed and treated with yellow ammonium sulphide to convert the white precipitate of copper thiocholine into a brown amorphous deposit of copper sulphide.

The whole mechanism of the thiocholine method has now been critically examined by Malmgren and Sylvén (1955) who were prompted to make their investigations when Zajicek, Sylvén and Datta (1954) found that the reaction product was deposited in the form of crystals. These had also been noted by Couteaux and Taxi (1952) and by Mohr and Gerebtzoff (1954). It appeared that these crystals of the initial reaction product must be dissolved and redeposited as the final product of copper sulphide. Analyses of the primary reaction product showed that it consisted of copper thiocholine sulphate and the following empirical formula was suggested :

$$[Cu^{+}—S—CH_2—CH_2—N^{+}(CH_3)_3]SO_4^{-}$$

It was observed to be slightly soluble at pH 8 but less soluble in acid media, and when a purified sample was placed on a coverslip and treated with dilute yellow ammonium sulphide the crystals dissolved and were replaced by an amorphous deposit of CuS. The authors considered that this was not topically related to the original crystals, that is to say there was no direct conversion of one to the other, *in situ*.

During the histochemical performance of the reaction minute rod-like crystals were deposited at presumed sites of cholinesterase activity. If the medium was saturated with regard to copper thiocholine (by the previous addition of this compound) the deposit was observed to grow into large needle-shaped crystals. Malmgren and Sylvén therefore considered that only the first deposits could be regarded as due to enzyme activity and that several factors other than enzyme activity must influence the amount of deposit at local sites.

These observations did not and do not diminish the usefulness of the thiocholine method for demonstrating AChE and ChE, but they suggest that localization is unlikely to be accurate at cytological levels unless the earliest small crystals are observed directly, without conversion to sulphide. Quantitative applications would seem to be impossible. Acting on the above suggestions, Holmstedt (1957b) has introduced a much modified version of the Koelle technique in which the medium is not saturated with copper thiocholine and in which conversion to copper sulphide is omitted. Details of this method, and also of Gomori's (1952) simplified version of Koelle's (1951) technique, appear in Appendix 16, p. 890.

Further theoretical criticisms of the principles of the thiocholine methods have come from Holt and Withers (1952) and from Ravin, Zacks and Seligman (1953). Both groups of authors condemned the

methods largely on account of the high charge on the molecule which they considered would lead to non-specific attachment to protein and to failure to penetrate barriers such as the red cell envelope. These criticisms are certainly valid, but nevertheless at its best (i.e. at pH 4·7 or below) the acetylthiocholine method gives almost exactly the same localization in the motor end plates of mammalian and reptilian muscle as does the best indoxyl method (Fig. 147, p. 472). In both cases the degree of localization is superior to, that is to say it looks better than the localization afforded by the best (most substantive) coupling azo dye method. In both cases the concentration of the final reaction product in the subneural apparatus of the motor end plate suggests that in addition to appearing better, the localization afforded really is more accurate.

While it is certain that displacement of the original precipitate of copper thiocholine sulphate occurs during conversion to copper sulphide, since in any case the degree of localization other than in the motor end plate cannot be regarded as cytologically accurate, it is probably better to tolerate the additional inaccuracy rather than to peer dubiously at the unconverted deposit. The one great advantage of the thiocholine methods, at the present time, is their specificity for AChE and ChE (see Figs. 152 and 153). If a suitable inhibitor for non-specific esterases can be found (see below), the employment of such an inhibitor in conjunction with one of the less specific substrates such as 4-chloro-5-bromoindoxyl, will doubtless be found preferable.

The Thiolacetic Acid Method

It was shown by Wilson (1951) that AChE could catalyse the hydrolysis of thiolacetic acid (CH_3COSH). This fact was made use of by Crevier and Bélanger (1953) who evolved a histochemical method for AChE using thiolacetic acid as substrate. The liberated H_2S was trapped by Pb^{2+} ions added as lead nitrate or acetate. The authors used either fresh frozen or cold formalin-fixed frozen sections mounted on slides and thoroughly dried to ensure adherence. They obtained clear localization of AChE in motor end plates, in neurones, and along certain fibre tracts in the C.N.S.

With this method I was able to reproduce the original authors' results, especially in fixed tissues, but with the usual incubation period (30–60 minutes at 22°) there was nearly always a non-specific deposition of lead sulphide which made the final result dirty. If the incubation period is kept short the results are somewhat cleaner. The method has probably not received the attention it deserves. References to it are scarcely to be found in the literature except in the field of electron histochemistry (see Chapter XXIX, p. 770). Details of the thiolacetic acid method are given in Appendix 16, p. 894.

Fig. 152. Rat brain. Medial habenular nucleus. Shows pseudocholinesterase in neurones after incubation with acetylthiocholine and 62.C.47. Koelle method. × 150.

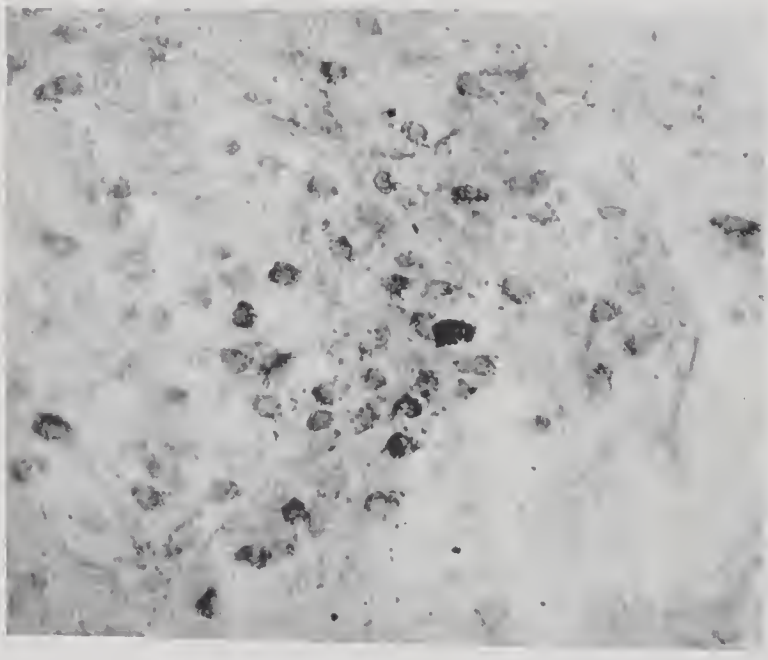


Fig. 153. As Fig. 152. Pseudocholinesterase after incubation with butyrylthiocholine. Koelle method. × 150.

[*To face p.* 480.

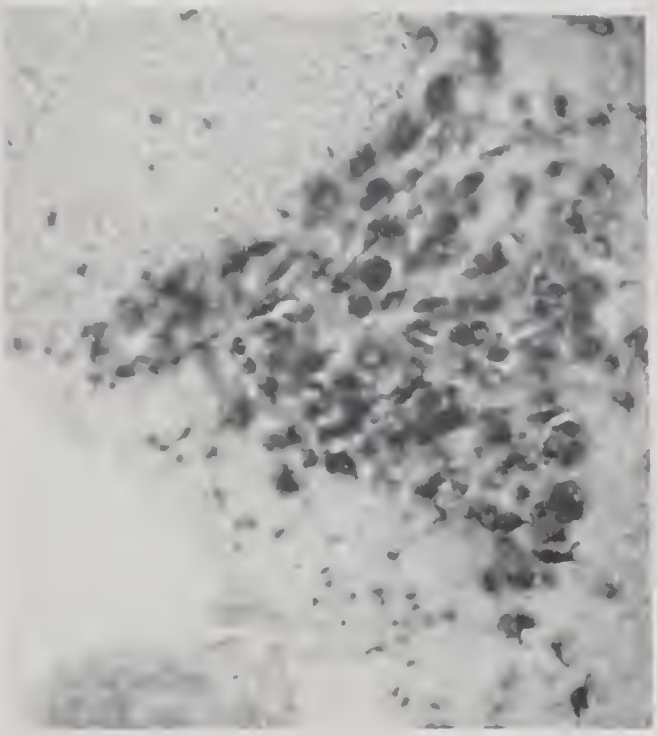


Fig. 154. Rat hypothalmus. Supraoptic nucleus. Shows staining in neurones and pericytes due to a spectrum of esterases. 5-Bromoindoxyl acetate. × 130.

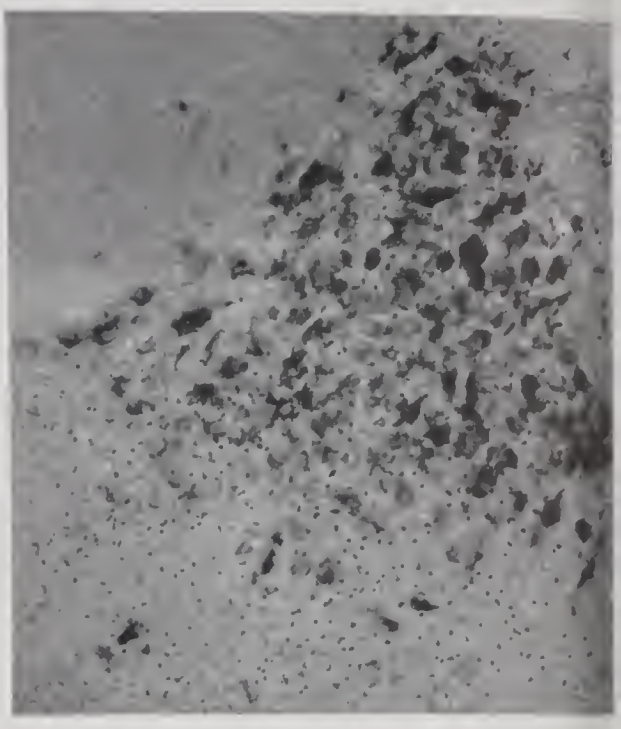


Fig. 155. As Fig. 154. Incubated in presence of 10^{-6} M—E 600. Shows general lowering of esterase activity except in pericytes. 5-Bromoindoxyl acetate. × 130.

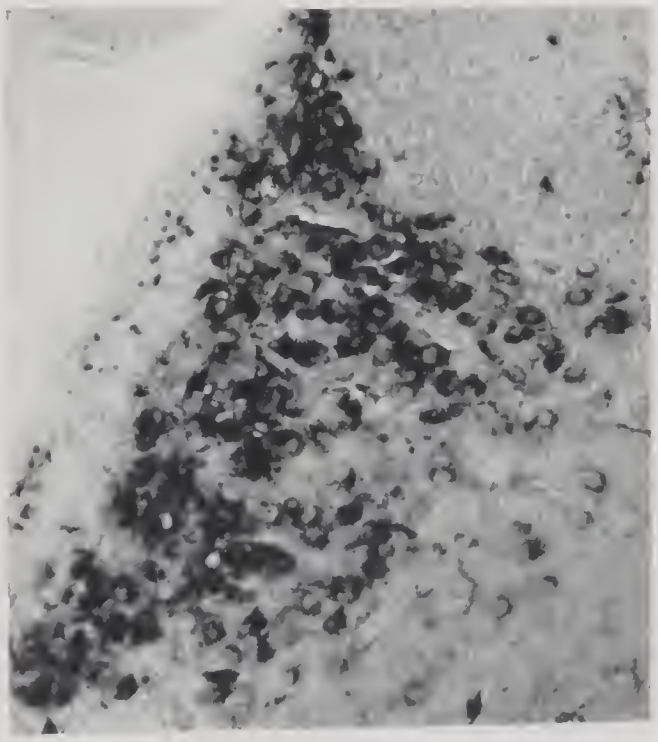


Fig. 156. As Figs. 154 and 155. Incubated in presence of 10^{-4} M– 62.C.47. Total esterase is scarcely affected. 5-Bromoindoxyl acetate. × 130.

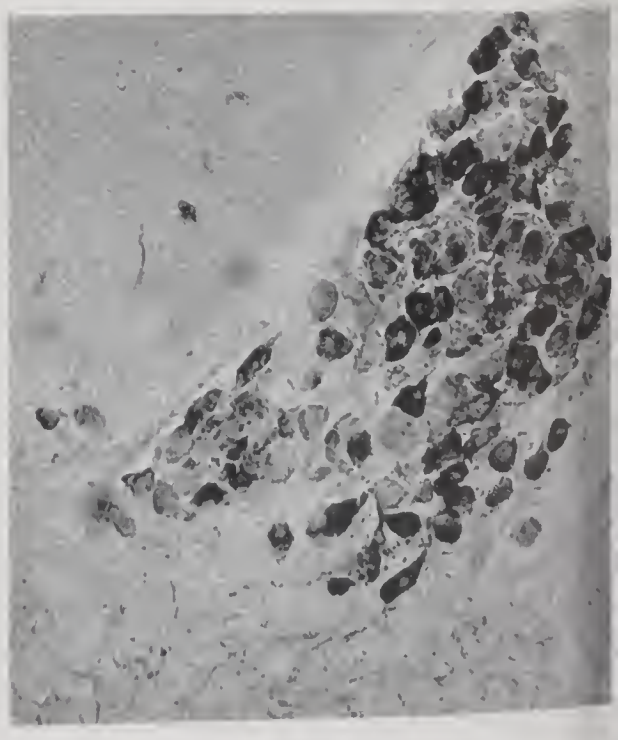


Fig. 157. Rat hypothalamus. Supraoptic nucleus in young male rat dehydrated by 14 days' treatment with 2·8 per cent. saline. Larger than normal cells contain greatly increased amounts of specific acetylcholinesterase. Koelle method. × 130.

Coupling Azo Dye Methods

The Nachlas-Seligman esterase method and its α-naphthylacetate and *o*-dianisidine modification can be used to demonstrate cholinesterases, since both ChE and AChE can hydrolyse acetates. So also can the Naphthol AS acetate methods (see Chessick, 1953). Adams (1949) found that a purified erythrocyte AChE hydrolysed *n*-butyl acetate and benzyl acetate at 49 and 68 per cent. of the rate of hydrolysis of acetyl β-methylcholine, respectively. Other authors agree with these results (Augustinsson, 1949 ; McNaughton and Zeller, 1949) and Underhay (1957) has shown that α-naphthyl acetate is hydrolysed by AChE and by ChE at a far higher rate than by the aliphatic and aromatic esterases. In tissues where non-specific esterase is absent the α-naphthyl acetate method can be used, with a single control section incubated in the presence of 10^{-6} M DFP, to demonstrate and distinguish between AChE and ChE. Fig. 148 shows the application of the method to unfixed frozen sections of rat diaphragm, in this case without inhibitor, so that the result indicates the sum of AChE and ChE. The fainter staining in the muscle fibres is attributable to non-specific esterase. Because of its simplicity the coupling azo dye technique might be supposed to be the method of choice for demonstration of cholinesterases as well as non-specific esterases but in practice the failure of the substrate to penetrate various lipid membranes (e.g. negative reaction with erythrocyte AChE in sections) makes the use of an alternative method imperative. Figs. 146 and 147 show rat intercostal muscle motor end plates by the *O*-acetyl-5-bromoindoxyl and *O*-acetyl-4-chloro-5-bromoindoxyl methods (Appendix 16, p. 888). Detail is much better than with the α-naphthyl acetate method.

Indoxyl and Substituted Indoxyl Methods

The high degree of sensitivity which characterizes these methods, and the ability of indoxyls to pass through lipid membranes, suggests that they will ultimately provide the method of choice for the demonstration of AChE. Holt (1952) showed that the rates of hydrolysis of indoxyl esters by AChE were comparable with those recorded for the corresponding choline esters, as shown in Table 37, which is taken from his paper.

Holt suggested that the failure of AChE to hydrolyse *O*-benzoylindoxyl might be used as a distinction between AChE and ChE, but its rate of hydrolysis by the latter is so slow that satisfactory conditions for precipitation in tissue sections are not achieved. Non-specific esterase hydrolyses this substrate rapidly so that a positive result is quickly obtained with sections of kidney, liver, etc. The precipitate is grossly crystalline, however. Further information with regard to the

TABLE 37

Hydrolysis of Various Esters by Cholinesterases

Substrate	AChE (Human red cell)	ChE (Human serum)
Acetylcholine	100	100
Acetyl-β-methylcholine	39	3
Benzoylcholine	2	116
O-acetylindoxyl	60	23
O-propionylindoxyl	41	63
O-butyrylindoxyl	12	33
O-benzoylindoxyl	0	3
O-acetyl-N-methylindoxyl	72	123

rates of hydrolysis of various esters has come from the studies of Underhay (1957). Her results are summarized in Table 38, below.

It can be observed that the relative rates of hydrolysis of the indoxyl esters by AChE are in the descending order acetate, propionate, butyrate (cf. Adams, 1949). For ChE, on the other hand, the order is butyrate, propionate, acetate for choline esters (cf. Adams and Whittaker, 1949) and propionate, butyrate, acetate for indoxyl esters.

TABLE 38

Relative Rates of Hydrolysis by Purified Cholinesterases.
(Expressed as percentage of rate of hydrolysis of acetylcholine)

Enzyme	Ester	Choline Esters	Indoxyl Esters	Phenyl Esters	α-Napthyl Esters
AChE	Acetate	100	70	107	53
	Propionate	84	22	62	—
	Butyrate	3	1	2	—
ChE	Acetate	100	30	54	96
	Propionate	260	72	141	—
	Butyrate	332	30	190	—

Inhibitors and Activators in Esterase Histochemistry

Before discussing the application of inhibitors and activators to the histochemical distinction of the various carboxylic esterases it is necessary to emphasize the very great differences which are observed between one species and another. Unless this is understood there may be disappointment when a given inhibitor fails to act as expected. The closest possible adherence to the results of biochemical studies is essential.

Distinction of Esterases from Lipases. Distinction between true lipases and non-specific esterases was at one time made by observing

the activation of the former by low concentrations of the salts of the bile acids, especially sodium taurocholate. Although an enzyme which is activated by this substance may be a true lipase, there is evidence that some types of non-specific esterase are similarly activated. The only reliable histochemical method of distinguishing the two groups is the use of unsaturated Tween-type substrates, as advocated by Gomori (1952b).

Distinction of Types of Non-specific Esterase. In an extensive series of histochemical studies Gomori and Chessick (1953a and b) found that the esterases of mammalian tissues showed marked organ and species specificities, the latter being very great for some tissues (e.g. brain) and much smaller for others (e.g. placenta). Gomori (1952a) divided the esterases into lipase, esterase, α-esterase, AS-esterase and cholinesterase. He continued (1952b) to use this classification, but later (1955), in the case of human esterases, proposed a division into hepatic, pancreatic and general types on the basis of histochemical substrate preference.

I do not consider that classifications of these types should be used by histochemists and I prefer to divide the esterases, following established biochemical practice, into A-, B-, and C-esterases. This can be done on the basis of inhibitor and activator studies such as those used by Pepler and Pearse (1957a) and by Hess and Pearse (1958). Table 39, below, indicates the lines on which histochemical differentiation between the three types of esterase can be attempted.

TABLE 39

Non-specific Esterases

(Esterases Insensitive to 2×10^{-6} M-Eserine)

Enzyme	Substrate	Complete Inhibition by	Activation by
A-esterase Arom-esterase Organophosphate-resistant esterase I	α-naphthyl acetate Naphthol AS acetates Indoxyl acetates	E 600, 10^{-2} M $CuSO_4$, 10^{-3} M* $Pb(NO_3)_2$, 10^{-3} M* $AgNO_3$, 10^{-2} M PCMB†, 10^{-4} M	Cysteine, 10^{-3} M
B-esterase Ali-esterase Organophosphate-sensitive esterase	α-naphthyl acetate Naphthol AS acetates Indoxyl acetates	E 600, 10^{-5} M Mipafox, 10^{-3} M Eserine, 10^{-4} M Ro3-0422, 10^{-7} M $AgNO_3$, 10^{-2} M	Cysteine, 10^{-3} M
C-esterase Organophosphate-resistant esterase II	Indoxyl acetates Naphthol AS acetates	β-phenylpropionic acid, 10^{-2} M $AgNO_3$, 10^{-2} M* $CuSO_4$, 10^{-3} M*	Phenylmercuric chloride, 10^{-4} M Trypsin, 1/2 sat. sol. PCMB†, 10^{-4} M

* Approximately 50 per cent. inhibition.
† *p*-chloromercuribenzoate.

In practice sections (usually cold formalin-fixed frozen sections) are incubated with 10^{-5} M-E 600 in buffer at pH 5·3 for one hour at 37°. B-type esterases are irreversibly inhibited by this treatment and, after washing, the sections are incubated by the chosen method to demonstrate the two organophosphate-resistant esterases. The method of choice is an indoxyl acetate method, preferably using 4-chloro-5-bromoacetylindoxyl as substrate.

Distinction between A- and C-esterases is made by following up the treatment with E 600 (or Mipafox) with one hour's incubation in buffer containing inhibitors or activators as indicated in Table 39. Thereafter the sections are incubated in substrate medium containing the chosen inhibitor or activator. The best compound for distinguishing A- and C- esterases seems to be *p*-chloromercuribenzoate though phenylmercuric chloride can be used as an alternative. At a concentration of 10^{-4} M these substances inhibit A-esterase but activate C-esterase to the extent of about 25 per cent. This effect is easily observable under histochemical conditions and Hess and Pearse (1958) showed that in rat kidney a moderate amount of the total indoxyl-esterase activity is due to a C-esterase.

Distinction of Cholinesterases. All the esterase substrates mentioned in this chapter can be hydrolysed by specific and non-specific cholinesterases and three of the methods described have been used extensively for the demonstration of these enzymes. They are the choline ester methods (usually myristoyl choline), the thiocholine methods and the indoxyl methods. Most popular have been the thiocholine methods, despite their obvious drawbacks, on account of their high specificity. Although a number of workers have used the specific substrates (acetylthiocholine, butyrylthiocholine) for distinguishing between the two enzymes, others, myself included, prefer to use a single substrate in conjunction with the specific inhibitors. As mentioned above, the provision of a selective method of inhibiting the non-specific esterases will enable the greatly superior indoxyl acetate methods to be used in place of the Koelle method for routine cholinesterase studies. In Table 40, below, are recorded the various inhibitors which have been, and are being, used in cholinesterase histochemistry.

As with the various non-specific esterase inhibitors, those listed in Table 40 are usually employed by pre-incubating sections for 1 hour in buffer before transferring them to the substrate medium. Reversible inhibitors are included in the latter as well as being used for pre-incubation but irreversible inhibitors are omitted. I believe that at the present time acetylcholinesterase is best identified by its ability to hydrolyse acetylthiocholine in the presence of the selective pseudocholinesterase *iso*OMPA (Pepler and Pearse, 1957a). In regions where significant concentrations of non-specific esterase are absent, as in the

TABLE 40

Cholinesterases

(Esterases Sensitive to 10^{-5} M-Eserine)

Enzyme	Substrate	Complete Inhibition by
All cholinesterases	α-naphthyl acetate Acetylthiocholine *O*-acetylindoxyls Myristoylcholine	Eserine, 10^{-5} M Ro3-0422, 10^{-8} M E 600, 10^{-7} M DFP, 10^{-5} M
Acetylcholinesterase Acetocholinesterase Specific cholinesterase True cholinesterase	α-naphthyl acetate Acetylthiocholine *O*-acetylindoxyls	DFP, 10^{-5} M Nu-683, 10^{-4} M Nu-1250, 10^{-8} M 62C47, 10^{-5} M 285C51, 10^{-4} M 284C51, 10^{-5} M
	Myristoyl choline	Poorly hydrolysed
	Butyrylthiocholine	Very slowly hydrolysed
Pseudocholinesterase Propionocholinesterase Butyrocholinesterase	α-naphthyl acetate Acetylthiocholine Butyrylthiocholine Myristoylcholine *O*-acetylindoxyls	DFP, 10^{-6} M Nu-683, 10^{-8} M Nu-1250, 10^{-5} M *iso*OMPA, 10^{-6} M

caudate nucleus for instance, the indoxyl acetate methods can equally well be employed. In the supraoptic and paraventricular nuclei of the hypothalamus, on the other hand, non-specific esterases are highly active and only the acetylthiocholine method gives a true appreciation of changes in AChE. This point is illustrated in Figs. 154 to 157, p. 481. In Fig. 154 the " total " indoxyl esterase of the supraoptic nucleus of rat hypothalamus is shown. Fig. 155 reveals that after obliteration of E 600-sensitive esterases considerable activity remains and Fig. 156 shows that after complete inhibition of AChE with 62C47 it is almost impossible to appreciate any change from the appearance of the control nucleus. The fourth illustration (Fig. 157) shows the enormously increased level of AChE (thiocholine method) which occurs during dehydration. The indoxyl esterase in this state shows an overall decrease, in spite of the raised level of AChE in the neurones.

Holmstedt (1957a) especially recommended Mipafox as the selective inhibitor of choice for ChE and 284C51 for AChE. As recorded earlier in this chapter, there is little to choose between the latter and 62C47. Both are more than 500 times as effective against AChE as against ChE. Mipafox, which was used as a selective inhibitor for ChE by Davison (1953) and by Denz (1954), is only 10 times as effective against this enzyme as against AChE. This is scarcely adequate. Judging by their I_{50} ratios lysivane and *iso*OMPA (see p. 461) are very

much superior to Mipafox as ChE inhibitors. Furthermore, the concentration of Mipafox with which Holmstedt observed complete inhibition of human serum ChE (4×10^{-6} M) was found by Pepler and Pearse (1957a) to have no effect on rat brain ChE. Little effect was observed, in fact, with concentrations as high as 10^{-4} M. Low concentrations of DFP are known to inactivate ChE, leaving AChE relatively unimpaired in most species (Adrian, Feldberg and Kilby, 1947 ; Hawkins and Mendel, 1947 ; Adams and Thompson, 1948), but this compound has not found favour for the purpose in the eyes of histochemists. Hawkins and Gunter (1946) tested a neostigmine analogue (Nu-683) and found that it gave marked selective inhibition for ChE while Hawkins and Mendel (1949) showed that a similar selective inhibition of AChE could be obtained with Nu-1250, a substituted trimethyl ammonium salt like Nu-683. Both these compounds are effective in histochemical use, but the margin of safety is less than with *iso*OMPA and 62C47 and I recommend the use of these last two inhibitors unreservedly.

Applied Esterase Histochemistry

The majority of applied studies in the esterase field have been concerned with the cholinesterases and the Koelle technique has been almost exclusively employed for this purpose. The list of works which appears below contains some of the more important applications of cholinesterase histochemistry, but it is not intended to be in any sense complete.

General Studies. Gerebtzoff *et al.* (1954), survey of cholinesterases ; Röhlich (1956), AChE after embedding in polyethylene glycols.

Electrophorus (*Electric Eel*). Couceiro *et al.* (1953) ; Couceiro, d'Almeida and Miranda (1955).

Salivary Glands. Snell and Garrett (1956). *Sweat Glands.* Hurley and Mescon (1956a).

Motor End Plates and Muscle. Couteaux (1951) ; Hellmann (1952), Harris (1954), with good list of references, including Russian ones ; Mohr and Gerebtzoff (1954) ; Dumont and Drouin (1954) ; Gerebtzoff (1954a) ; Coers (1954), amyotonia congenita ; Snell and McIntyre (1956) ; Durante (1956, 1957) ; Zelená and Szentágothai (1956–57) ; Lundin (1958).

Synapses and Nerve Endings. Kupfer (1951) ; Szentágothai *et al.* (1954–55) ; Hebb and Hill (1955) ; Hurley and Mescon (1955, 1956b) ; Koelle (1955b) ; Bergner (1957). *Retina.* Francis (1953) ; Hebb *et al.* (1953) ; Leplat and Gerebtzoff (1956). *Liver.* Gerebtzoff (1954a).

Blood and Bone Marrow Cells. Zajicek *et al.* (1954) ; Davies and Rutland (1956) ; Rogister (1956).

FIG. 159. Formalin-fixed frozen section (10 μ) of guinea-pig adrenal. To show apparent concentration of β-glucuronidase in the inner zona reticularis. Coupling azo dye method using 8-hydroxyquinoline glucuronide. $\times$ 9.

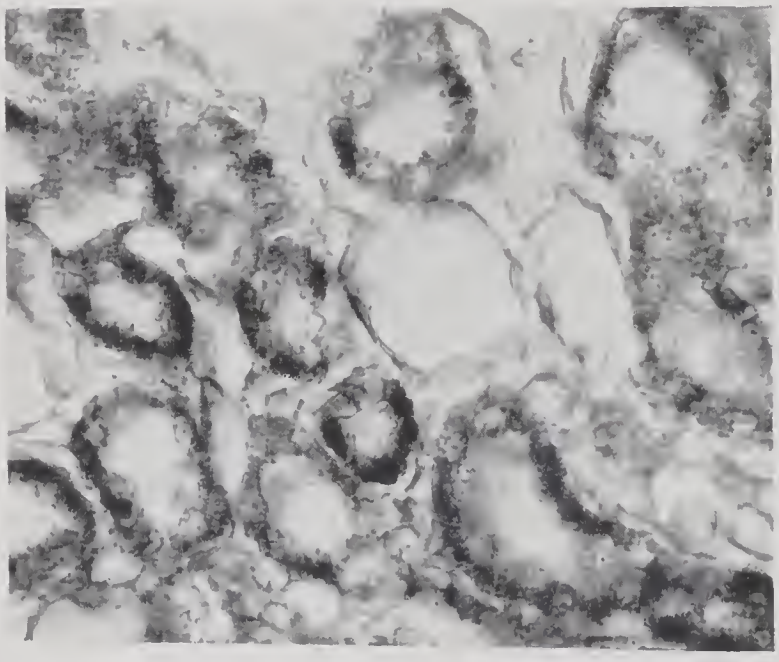


FIG. 160. Rat kidney. Shows localization of β-glucuronidase. Post-coupling method. $\times$ 400.

[*To face p.* 486.

FIG. 161. Rat cerebellum. Shows β-glucuronidase in cytoplasm of Purkinje cells. Ferric hydroxyquinoline method (Fishman-Baker). × 550.

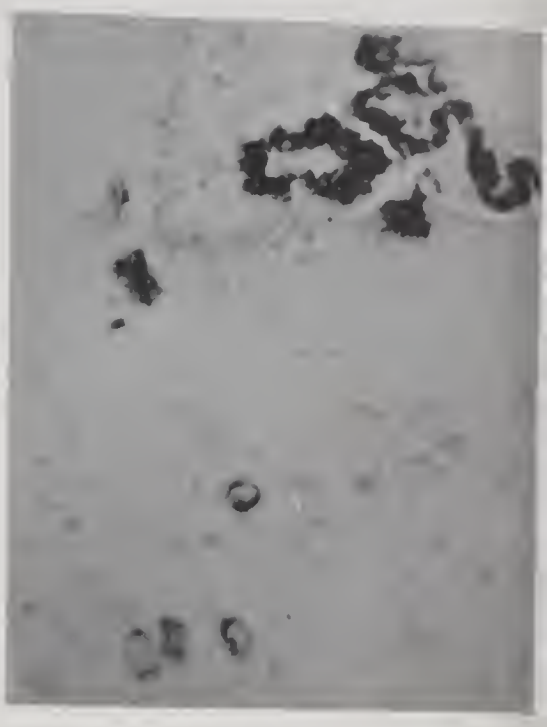


FIG. 162. Rat brain. Choroid plexus. Shows strong β-glucuronidase activity, present also in some of the neurones. Ferric hydroxyquinoline method. × 250.

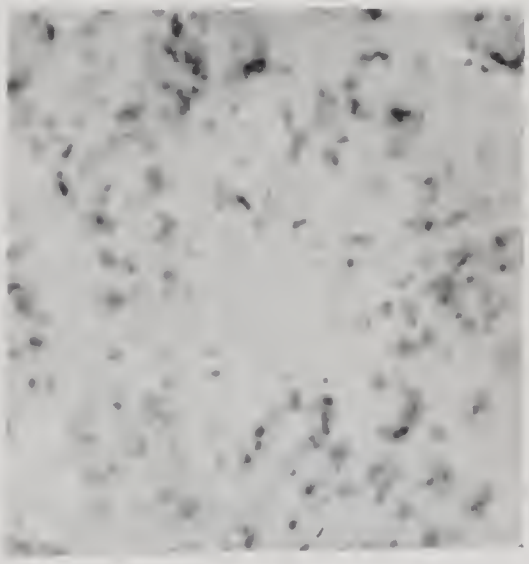


FIG. 163. Rat kidney. Briefly fixed in chloral-formalin. Shows localization of β-glucuronidase activity in particles, some of which resemble mitochondria. Ferric hydroxyquinoline method. × 1,250.

Central Nervous System. Hull (1954) ; Koelle (1954, 1955a) ; Csillik and Savay (1954) ; Abrahams *et al.* (1956), biochemical study ; Giacobini and Zajicek (1956) ; Hardwick and Hebb (1956) ; Pepler (1958), miracidium of *Schistosoma mansoni*. *Placenta.* Gerebtzoff (1957). *Thyroid.* Dumont (1956) ; Pepler and Pearse (1957b). *Skin.* Magnus and Thompson (1954), biochemical study ; *Adrenal.* Hagen (1955) ; Allen *et al.* (1957).

Studies of the non-specific esterases and lipases have been far less frequently reported in the literature, though the α-naphthyl acetate methods have, in fact, been widely employed in general studies of enzyme histochemistry in various physiological and pathological states. The list of references given below, again incomplete, refers to papers largely or wholly concerned with the non-specific esterases and lipases.

General Studies. Nachlas and Seligman (1949b) ; Bernsohn *et al.* (1954) ; Curri (1954) ; Malaty and Bourne (1954) ; Verne (1954).

Central Nervous System. Gomori and Chessick (1953) ; Savay *et al.* (1953). *Embryos.* Zachs (1954) ; Reale (1955) ; Sakae (1955) ; Rossi, Pescetto and Reale (1957). *Skin.* Wells (1957). *Spléen.* Argyris (1956). *Liver.* Underhay *et al.* (1956) ; Svanborg (1954.) *Kidney.* Holt and Withers (1952) ; Pearse (1953) ; Holt (1956) ; Hess and Pearse (1958). *Hypophysis.* Fand (1955) ; Pearse (1956). *Thyroid.* Weber (1954). *Connective Tissues.* Steigleder and Löffler (1956a and b). *Salivary Glands.* Hill and Bourne (1954) ; Burstone (1956). *Lipofuscins.* Gedigk and Bontke (1956). *Blood Cells.* Wachstein and Wolf (1958).

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CHAPTER XVII

GLYCOSIDASES AND TRANSGLYCOSYLASES

Introduction

Two groups of enzymes are dealt with in this chapter. The first, the glycosidases, contains such enzymes as the α- and β-amylases, amylo-1, 6- α-glucosidases, lysozyme, neuraminidase, β-acetylamino-glucosidase, α- and β-glucosidases and galactosidases, and the β-glucuronidases. From the histochemical point of view the most important is the last of these but methods exist for four others, α- and β-glucosidase, β-galactosidase and acetyl-β-glucosaminidase.

The second group contains a large number of enzymes capable of transferring glycosyl groups and some of these can build up long chain polymers of the type used in vertebrate and invertebrate tissues for carbohydrate storage and for structural purposes (starch, dextran, chitin). Hyaluronidase and nucleoside phosphorylase belong to this group, as do glucosan phosphorylase and glucosan transglycosylase. These last two enzymes, known familiarly as P and Q enzymes respectively, can be demonstrated histochemically.

The Glycosidases

Biochemistry of the β-glucuronidases

The β-glucuronidases of animal and invertebrate tissues are a group of enzymes with specificity for the β-glycoside linkage of a variety of naturally occurring and synthetic glucuronides. The earliest studies of any importance were made by Oshima (1934, 1936), using ox spleen and menthyl glucuronide as substrate. In recent years the β-glucuronidases have come to occupy an important position in the biochemical literature and this was adequately summarized by Fishman (1950). It has been suggested that the β-glucuronidases may have three functions in the body: (1) the conjugation of steroid hormones, (2) the hydrolysis of conjugated glucuronides, and (3) participation in cellular proliferation. Fishman (1940) was of the opinion that the β-glucuronidases played a synthetic role in the body and later (1947) he proposed that these enzymes catalyzed the conjugation of œstrogens with glucuronic acid, a process regarded as one of "metabolic conjugation", essential for the function of such steroids, and not simply a detoxication. Levvy (1947, 1948) took the opposite view, that the β-glucuronidases were concerned solely with the hydrolysis of glucuronides, and strong

evidence against Fishman's theory was given by Karunairatnam and Levvy (1949), who showed that saccharic acid, which inhibits β-glucuronidases, failed to influence the formation of glucuronides by mouse liver slices *in vitro*. Levvy, Kerr and Campbell (1948) related the β-glucuronidase activity of mouse liver and kidney to the degree of cellular proliferation which was taking place, and Kerr, Campbell and Levvy (1949) similarly showed that uterine β-glucuronidase, in the mouse, was an index of the growth of that organ. Hollinger and Rossiter (1952), in their studies on regenerating nerve, found a peak of cellular proliferation (DNA) at 4 days and a peak enzyme activity at 16 days. At the same time Fishman and his co-workers (Fishman and Anlyan, 1947 ; Fishman, Anlyan and Gordon, 1947 ; Fishman and Bigelow, 1950) reported increased amounts of β-glucuronidase in cancerous tissues which they regarded as characteristic of neoplasia. Mills and Smith (1951), on the other hand, believed that the raised β-glucuronidase activity of tumours was related to a change in cell type rather than to the neoplastic change itself. They showed that rat hepatomas produced by feeding dimethylaminoazobenzene had a lower β-glucuronidase activity than normal rat liver.

These differences of opinion have certainly not yet been resolved. Pellegrino and Villani (1956) observed a rise in β-glucuronidase in lymphatic tissues during fasting, a process accompanied by marked atrophy of the tissues concerned. A later study by these authors (1957) showed a similar atrophy and a similar rise in β-glucuronidase in tissues following irradiation. Pellegrino and Villani considered that their results indicated a correlation between atrophy and β-glucuronidase activity. Further work designed to test the cellular proliferation hypothesis of Levvy and his associates was undertaken by Mills, Paul and Smith (1953b). These authors measured enzyme activity and DNA concentration in rat liver from the 16th day of embryonic life until the 220th day of adult life. From a very low level at birth activity rose to a maximum between the 25th and 30th days, falling slightly thereafter to reach a constant adult level. Using the allometric method of analysis (Huxley, 1924) they compared β-glucuronidase levels with DNA content and found two phases of activity. In the first phase the enzyme increased faster than the number of cells but in the second phase the two rates were identical. These results suggest a species difference in the behaviour of liver β-glucuronidase as between the mouse (Levvy *et al.*, 1948) and the rat (Mills *et al.*, 1953b). A study of β-glucuronidase activity during the early development of *Xenopus laevis* embryos was made by Billett (1957). He found that a slight fall in enzyme levels occurred at the time of formation of the neural plates but could find no evidence to connect β-glucuronidase with cell proliferation.

The most acceptable hypothesis still seems to be that of Fishman (1947), that β-glucuronidase activity is related to steroid metabolism. The observation by Levvy, McAllen and Marsh (1958) that the preputial gland of the female rat is the richest known source of β-glucuronidase is of considerable interest and it provides a convenient histochemical control tissue (see Fishman and Baker, 1956). The connection between atrophic changes and high β-glucuronidase levels is no more surprising than the rise of acid phosphatase which occurs under similar circumstances and which is apparently due to the release of enzyme from destroyed intracellular organelles.

Enzymes hydrolysing β-glucuronides have their optimum pH in the acid range. Mills (1948) described two glucuronidases in ox spleen, one having its optimum at pH 4·5, the other at pH 5·2, and Kerr *et al.* (1949) found both enzymes in mouse spleen but only the first in mouse uterus. Histochemical tests are usually carried out in the region 5·0–5·2 and no reaction is obtainable at pH 4·5, with any of the available methods. Since the original work of Mills it has become apparent that the overall activity of ox spleen extracts cannot be explained on the basis of two glucuronidases. Further work by Mills, Paul and Smith (1953a) has shown that there are three fractions, with optima at pH 3·4, 4·5 and 5·2 and examination of the effects of variation in substrate concentration on reaction velocity, and other considerations, allow the conclusion that the three fractions can be considered as separate enzymes. There still remains, obviously, a wide gulf between biochemical and histochemical studies in the field of mammalian β-glucuronidases.

In contrast to the mammalian enzymes the β-glucuronidase of the limpet (*Patella vulgata*), has a broad pH optimum in the region of pH 4·0 (Dodgson *et al.*, 1953) and the position of the peak can be altered, without marked change in optimum activity, by varying the concentration of buffer or substrate (Smith and Mills, 1953; Levvy, 1954; Levvy, Hay and Marsh, 1957).

Activators and Inhibitors. The earliest known inhibitors of the β-glucuronidases were organic acids such as citric acid (Oshima, 1936). Later Karunairatnam and Levvy (1949) showed that mucic and saccharic acids could inhibit the enzyme and that D-saccharic acid, the stronger of the two, acted competitively at pH 5·2. Becker and Friedenwald (1949) found that ascorbic acid and heparin were glucuronidase inhibitors and Spencer and Williams (1951) demonstrated inhibition of the enzyme by glucuronate at pH 5·2.

In their comprehensive study (1953a) Mills and his associates found that citric acid would, in fact, inhibit only the two enzymes active at pH 3·4 and 4·5. This is shown in Fig. 158, below.

The most effective organic acids were saccharate (10^{-4}M) mucate

(10^{-3}M) and oxalate (10^{-2}M), in that order but Levvy (1952) observed that saccharo-1:4-lactone was more effective than saccharate itself as a competitive inhibitor for β-glucuronidase. Later Karunairatnam and Levvy (1949) showed that the high activity of saccharate was due to traces of the lactone present in most samples. Ascorbic acid is a non-competitive inhibitor for all three mammalian enzymes, a concentration of 10^{-3}M causing approximately 50 per cent. inhibition in each case.

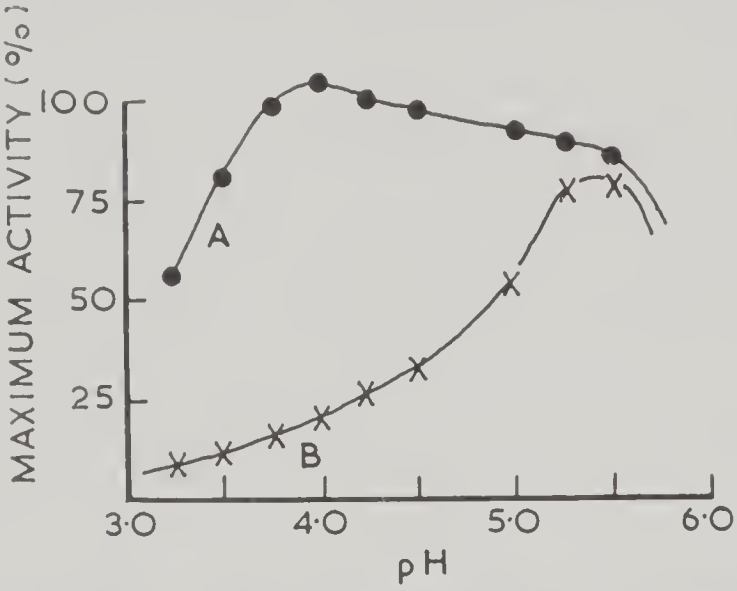


FIG. 158. pH Activity Curves of Ox Spleen Extract in Acetate Buffer (A) and Citrate Buffer (B)

Another inhibitor, described by Wills and Wormall (1950) is the polysulphonic acid trypanocide suramin. This is inhibitory in low concentrations at pH 4·5 but practically without activity at pH 5·2. Above this level activation is observed. Inhibition by suramin is non-competitive. Mills *et al.* (1953a) observed that 15 per cent. formaldehyde caused only 15 per cent. inhibition of the fractions with optima at pH 4·5 and 5·2. Activation of the enzyme by phthalate was noted by Smith and Mills (1953), and a specific activation of purified spleen β-glucuronidase by DNA was reported by Bernfeld, Guarino and Fishman (1950). Later work, summarized by Levvy, McAllan and Marsh (1958), has shown that β-glucuronidase at high dilutions requires the presence of proteins for full activity. They do not regard DNA as a satisfactory substitute for protein since its activity is influenced by other tissue constituents and also by the pH.

Methods for β-glucuronidases (DW. 174)

In contrast to the volume of biochemical work on the β-glucuronidases, relatively little has been done histochemically towards their localization in the tissues, although four types of method have been suggested or practised for the purpose. These methods fall into the

four main divisions of Table 41, where they are classified as: (1) *non coupling azo dye* methods; (2) *simultaneous coupling azo dye* methods ; (3) *post-coupling azo dye* methods ; and (4) *non-azo dye*, or other, methods. In the case of the first three the prefix refers to the time at which coupling with a diazonium salt is performed. These terms have

TABLE 41

Histochemical Methods for β-glucuronidases

Type of method	Substrate (glucuronide)	Precipitant	Demonstrator	Author
Non-coupling azo	1-*o*-hydroxy-phenyl azo-2-naphthyl	Nil	Nil	Friedenwald and Becker (1948)
Simultaneous coupling azo dye A	1 and 2 naphthyl	*o*-dianisidine (diazo)	Nil	Seligman *et al.* (1949)
Simultaneous coupling azo dye B	8-hydroxy-quinolyl	4-benzoyla-mino-2:5-dimethoxy-aniline (diazo)	Nil	Pearse (unpublished)
Post-coupling azo dye	6-bromo-2-naphthyl	Nil	*o*-dianisidine (diazo)	Seligman and Nachlas (1950)
Non-azo dye	8-hydroxy-quinolyl	Ferric ions	Perls' reagent	Friedenwald and Becker (1948) Fishman and Baker (1956)
	5-bromoindoxyl	Nil	Nil	Holt (unpublished)

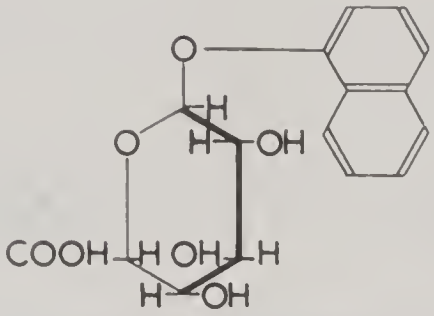
already been used in Chapters XIII to XVI with reference to the azo dye methods. In the fourth case, the prefix is self-explanatory.

Non-coupling Azo Dye Method

This method, the first of two described in the same paper by Friedenwald and Becker (1948), comes under the heading of a non-coupling azo dye technique. It employed as substrate the glucuronide of a water-insoluble azo dye, 1-*o*-hydroxyphenylazo-2-naphthol, and the sites of glucuronidase activitiy were supposedly indicated by the precipitation of a bright red dye. Campbell (1949), however, showed that there was no appreciable difference in staining intensity between active control sections and sections previously incubated with, or incubated in the presence of, the specific inhibitor saccharic acid. Burton and Pearse (1952) confirmed this finding and showed additionally (1) that with heat-inactivated sections incubation with the substrate produced results comparable with those in active controls, and (2) that in active or inactivated sections a dilute alcoholic solution of the reaction product (1-*o*-hydroxyphenolazo-2-naphthol) produced a similar result. This method does not localize, or even necessarily demonstrate, β-glucuronidase in the tissues.

Simultaneous Coupling Azo Dye Methods

Naphthyl Glucuronides. Seligman *et al.* (1949) reported attempts to localize β-glucuronidase using the above principle and 1- or 2-naphthyl glucuronide as substrate. Although these authors stated that hydrolysis of 1-naphthyl glucuronide was too slow to allow the establishment of a histochemical technique, I found that it was possible to obtain coloured precipitates, using this substrate at pH 5·0 and 37° for 4–16 hours, with a variety of diazonium salts. In no case was the result satisfactory from the histochemical point of view. Seligman reported a similar lack of success with 2-napththyl glucuronide. The formula for 1-naphthyl glucuronide, which appears below, indicates the general structure of the phenolic glucuronides used in biochemistry and histochemistry.



1-naphthyl glucuronide

8-hydroxyquinoline Glucuronide. Burton and Pearse (1952) reported the largely unsuccessful use of this substrate in a coupling azo dye technique with a variety of diazotates. Further experiments showed, however, that the diazotate of 4-benzoylamino-2 : 5-dimethoxyaniline was particularly stable at pH 5·0 and it can be used at this pH for prolonged incubation at 37° or even at 60° for some hours. Using approximately 0·005 M-8-hydroxyquinoline glucuronide in 0·1 M-acetate buffer as substrate, cold formalin-fixed frozen sections of various animal tissues were incubated for 2–4 hours at 37° in the presence of 1 mg./ml. of the diazonium salt. A bright orange-red granular precipitate, and occasionally smaller amounts of a larger flocculent precipitate, were observed in tissues known to contain β-glucuronidase, or shown to do so by an alternative method (see below). Sections incubated in the presence of 0·001 M-saccharate invariably showed no precipitate of either kind.

Localization was in a few tissues excellent, but in the majority unsatisfactory. In rabbit spleen the granules were constantly associated with small groups of reticulum cells and in the guinea-pig adrenal (Fig. 159) they were largely restricted to the inner zona reticularis of the cortex. They were also present to a small extent throughout the

medulla but absent from the rest of the cortex. The method is not yet sufficiently reliable for application to diagnostic histochemistry, but if control of the rate of precipitation and of precipitate size can be obtained by the use of suitable diazonium salts it may become valuable because of its simplicity.

Post-coupling Azo Dye Methods

Because of the unsatisfactory results which they obtained with the simultaneous coupling method, Seligman *et al.* (1949) made use of the post-coupling azo dye principle. For this purpose they used the glucuronide of 6-bromo-2-naphthol, made biosynthetically by administration of the naphthol compound to rabbits. Unlike the majority of the glucuronides, this particular example can be obtained in an impure state by continuous ether extraction of the acid urine, followed by solution of the dried extract in hot water and its precipitation from this as the *p*-toluidine salt. Seligman, Tsou, Rutenburg and Cohen (1954), however, have shown that the yield obtained is very poor and biosynthesis is thus unsuitable for the preparation of 6-bromo-2-naphthyl glucuronide. They have prepared the compound in the pure state by oxidation of the β-D-glucopyranoside with oxygen in the presence of a platinum black catalyst.

Criticism of the post-coupling method by Burton and Pearse (1952) was criticized in its turn by Seligman *et al.* (1954), with some justification. It is now evident that the substrate which we used contained only a small amount of 6-bromo-2-naphthyl glucuronide and most of the diffusion and non-specific staining which we observed was due to the presence of non-halogenated naphthyl glucuronides.

In the technique developed by Seligman and his associates, which is given in detail in Appendix 17, p. 897, fresh cold microtome sections are post-fixed for short periods in cold formalin before incubation with the substrate in 5 per cent. methanol at pH 4·95. After incubation they are washed and then treated with Fast Blue B (6) at pH 7·5. The colour of the final product varies between bluish-purple and pinkish-red. On storage progressive deterioration takes place and the blue product diffuses and dissolves in lipids to give a reddish colour. With this method (Fig. 160, p. 496) the main difficulty seems to be due to diffusion of enzyme from the inadequately fixed sections. If cold microtome sections are used these should be fixed in Fishman and Baker's (1956) chloral hydrate formalin at 0-4° for at least an hour before incubation. Improved localization is produced by the addition of PVP or methyl cellulose but in both cases the reaction proceeds more slowly.

The post-coupling azo dye methods for β-glucuronidase have been little used in applied histochemistry, partly on account of difficulties in

obtaining the substrate. In my hands, though the overall picture resembles that given by the modified Friedenwald-Becker technique (see below), localization is never as sharp as it is with the latter. In areas of weak activity, or in tissues containing much lipid, the red colour of the final product is confusing. Moreover, I have not been able to produce mitochondrial or lysosomal localization of the enzyme such as can be seen in protected material incubated by the Friedenwald-Becker method. According to biochemical evidence β-glucuronidase is both mitochondrial and microsomal but such a localization of this soluble enzyme is not necessarily valid.

The Ferric Hydroxyquinoline Method

The second method produced by Friedenwald and Becker (1948) for the demonstration of β-glucuronidase depended on the hydrolysis of 8-hydroxyquinoline glucuronide, by unfixed tissue sections, in the presence of a ferric salt; this combined with the liberated 8-hydroxyquinoline to form an insoluble greenish-black precipitate of ferric hydroxyquinoline. The latter was subsequently converted to Prussian blue by means of Perls' acid-ferrocyanide reagent, partly to enable permanent preparations to be made by converting it into an alcohol insoluble compound, and partly because the initial greenish-black colour was poorly visible under the microscope. In the performance of the reaction several complicating factors were found. The incubating medium had to be freshly prepared on the day of use and immediately prior to use it had to be filtered in an incubator at 37°. Campbell (1949) found that unless a very fine filter was used (Whatman No. 40 paper), and double filtration carried out, trouble was still experienced with a fine suspension of ferric hydroxyquinoline, which tended to precipitate all over the slide. The original authors observed that diffusion of the ferric hydroxyquinoline reaction product caused a precipitate which covered the whole surface of the slide, but this was overcome, according to their statements, by saturating the incubating medium with the reaction product before starting. The incubation time was given as 3–18 hours and for most tissues the longer period was required. Since 8-hydroxyquinoline glucuronide is rapidly hydrolysed by spleen β-glucuronidase *in vitro*, and in the coupling azo dye method described above, the long incubation period necessary with the present method must be due to inhibition of the enzyme by ferric salts. Many other metallic ions were tried by the original authors as capture reagents for 8-hydroxyquinoline but only ferric ions gave a satisfactory result.

A critical study of the ferric hydroxyquinoline method was made by Burton and Pearse (1952). Initial experiences with the method were disappointing since it appeared capricious and at best capable

of giving only very coarse localization of enzyme activity. Despite saturation of the substrate solution with ferric hydroxyquinoline, as recommended by the original authors, surface precipitation was found to be troublesome. The preparations appeared dirty as though the precipitate had been deposited at random.

Modification of the Original Method. Two principal alterations were made to the original method. First, frozen sections of tissues fixed in cold formalin (4°), for 4–12 hours, were substituted for unfixed sections and secondly, ferric sulphate was used instead of ferric chloride in the incubating medium. These modifications, together, had two important effects: (1) they reduced non-specific precipitation to a minimum, and (2) they reduced the size of the final precipitate by about 50 per cent. The use of formalin fixation considerably widened the range of tissues to which the method could be applied and the substitution of $Fe_2(SO_4)_3$ for $FeCl_3$ enabled the incubating medium to be made up in bulk and stored at 4° for at least ten days without deterioration. This proved to be a considerable convenience in practice. The specificity of localization was controlled either by using sections incubated in the absence of substrate, as in the original authors' technique, or by treating other sections with the specific inhibitor (0·001 M-saccharic acid). Using this inhibitor Campbell found that the hydrolysis of 8-hydroxyquinoline glucuronide was almost entirely prevented, judging by the lack of a histochemical reaction, and these results have been amply confirmed, complete inhibition being regularly obtained both in sections and in the test-tube.

The results of the application of the method to rat kidney are shown in Plate VIA. At low magnifications it was observed to give clear, and apparently satisfactory localization. With higher magnifications the precipitate of Prussian blue was still found to be coarsely granular, and accurate intracellular localization of the enzyme was limited on account of this factor.

Further Modifications by Fishman and Baker. The whole aspect of the Friedenwald-Becker method has been altered by the very thorough studies of Fishman and Baker (1956) whose modification of the method is given in full in Appendix 17, p. 895. A capricious and uncertain technique has been converted into one of absolute reliability which can be used with confidence for studies in the applied histochemistry of the glucuronidases.

The Fishman-Baker procedure differs from earlier methods in several important essentials. The chloral-formalin fixative which is employed certainly leads to increased preservation of the enzyme in desmo form (see Chapter XIII, p. 382) but there is still uncertainty as to the mechanism by which chloral exerts its effect. A very large number of alternative fixatives were tested by the authors but with the majority

the incubation period was prolonged and the size of the final precipitate too large for accurate localization. In particular, cold formalin fixation was observed to lead to non-uniform deposition of the ferric hydroxyquinoline precipitate. The incubation medium has been modified in several respects, the most important of which is probably the addition of gum acacia. It is now stable in the cold for long periods. The main innovation of the new procedure, however, is the introduction of a period of pre-incubation at 8°, the sections being maintained in the incubating medium at this temperature for 17–24 hours. Subsequently they are incubated for a relatively short period at 37°.

I have tested the Fishman-Baker procedure on chloral-formalin fixed frozen sections, as advised by the authors, and find it entirely reproducible as far as rat tissues are concerned. Illustrations of the results of the method are given in Plates VIB and C, p. 512. The sharpness of localization is very evident and the precipitate, in most tissues, is microcrystalline. The method can also be used with mounted cold microtome sections (Figs. 161 and 162, p. 497) if these are post-fixed in cold chloral-formalin for 1–4 hours. Unfixed cold microtome sections can be employed if sufficient sucrose, dextran or PVP is included in the medium. In preparations treated in this way it is possible to localize the enzyme within intracellular structures resembling mitochondria (Fig. 163, p. 497). If unfixed frozen sections are used without post-fixation, or in a non-protective medium, widespread diffusion of the final product is observed, presumably due to diffusion of enzyme.

Application of the Ferric Hydroxyquinoline Methods. Modifications of the method have been used by a number of workers. Hayashi and his associates (1955, 1956–57) have studied changes in the distribution of uterine β-glucuronidase during the oestrus cycle in the rat. Activity was noted to be low in pro-oestrus. In early oestrus a rise in activity occurred in the basal layers of the endometrium and in late oestrus high activity was found in the surface and glandular epithelium. After oestrus activity declined to a minimum level in di-oestrus.

The localization of β-glucuronidase in the digestive gland of the Roman snail was studied by Billett and McGee-Russell (1955) and later (1956) these authors studied the distribution in newt liver. They found, especially in the latter case, that the precipitate was coarse and macrocrystalline after 1–4 hours' incubation and considered that the final stage of conversion to Prussian blue was unnecessary and dangerous. Most workers would probably disagree with this last conclusion. Billett and McGee-Russell also noted that saccharate (0·0005 M) failed to inhibit the enzyme in newt liver, and in mouse liver and kidney, when the pH was below 4, although it did so in other mammalian tissues and in snail digestive gland. They felt that the normal concentration of Fe^{3+} ions in the medium was inhibitory to the enzyme and that by

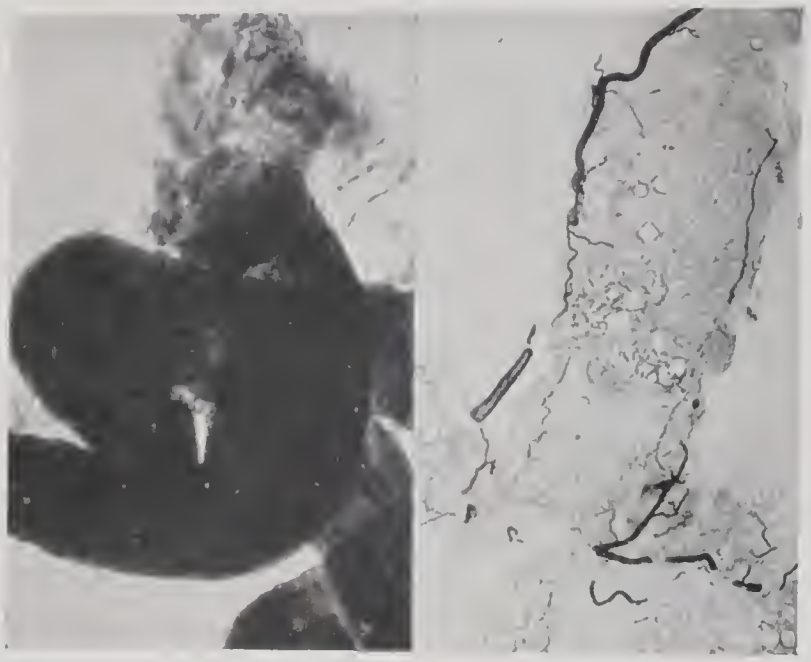


FIG. 164. Cockroach (*Periplaneta americana*). Right and left colleterial glands. Strong β-glucosidase activity in the right gland with only traces of activity in the left gland. Post-coupling method. × 75.

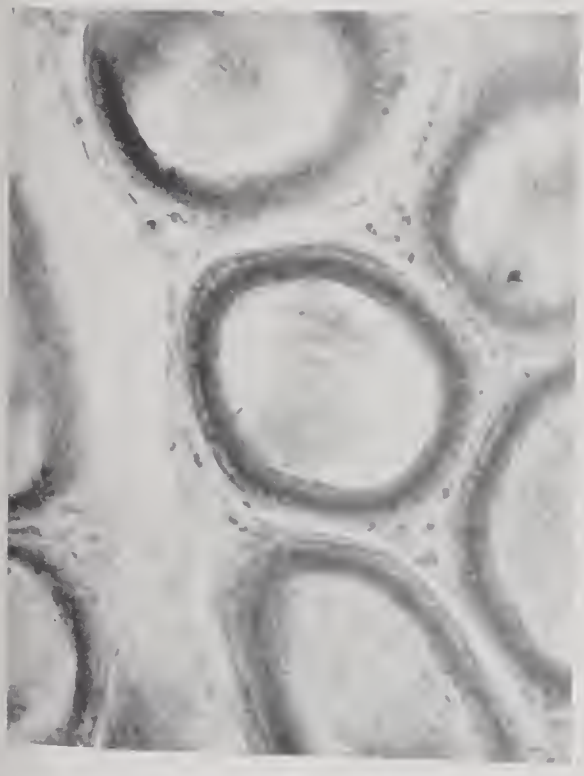


FIG. 165. Rat. Epididymis. Strong activity of acetyl-β-glucosaminidase. Coupling azo dye method. × 136.

FIG. 166. Rat. Skeletal muscle. Phosphorylase activity confined to the A-bands. Takeuchi method. × 500.

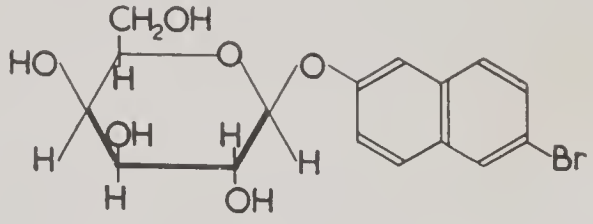
[*To face p.* 500.

reacting with Fe^{3+} saccharate might cause an apparent activation. It was concluded that the Ferric hydroxyquinoline method could not give a quantitative appreciation of enzyme activity since Fe^{3+} adsorption might vary greatly between different tissues and cells. The necessary critical studies of the method have yet to be made.

Methods based on Indoxyl Glucuronides. Although no reports have yet appeared in the literature the synthesis and histochemical utilization of indoxyl glycosides has been undertaken in several different laboratories. Little is to be expected from the use of unsubstituted indoxyl glycoside (see Chapter XVI, p. 469), but the halogen-substituted indoxyl glycosides have great promise as histochemical substrates. Until a suitable indoxyl glucuronide becomes available, however, it will be necessary for the majority of workers to continue to use the ferric hydroxyquinoline method or the post-coupling method of Seligman, Tsou, Rutenburg and Cohen (1954).

Method for β-galactosidase (DW. 163)

A post-coupling azo dye method for β-galactosidase has recently been evolved by Seligman and his associates (Cohen *et al.*, 1952a) using 6-bromo-2-naphthol-β-D-galactoside as substrate.



This compound was found to be hydrolyzed six times faster than 2-naphthyl galactoside, by the enzyme present in tissues such as rat kidney, and a method for *in vitro* estimation was based on its use.

In the production of a histochemical method a number of difficulties were encountered and reported. Enzyme activity was initially low and inhibition by diazonium salts prevented the establishment of a simultaneous coupling technique. Acetone-fixed paraffin sections contained little enzyme and formalin-fixed frozen sections less than 25 per cent., so that fresh frozen sections had to be employed. An attempt was made to prevent diffusion of the enzyme, which was observed to be considerable, by incubation in the presence of 1 M-NaCl. In the method finally evolved the mounted sections were incubated for 2 hours at 37° and pH 4·95 with the substrate and 1 M-NaCl. They were then washed and coupled with diazotized *o*-dianisidine at pH 7·5, with the production of a bluish-purple final product.

A later report by Rutenburg *et al.* (1958b) gives details of the further development of the method. A return has been made to the use of cold

formalin-fixed seetions and these are incubated, free-floating, for 2-4 hours at 37°. After coupling with the diazonium salt (fast blue B is still recommended) the final product is a purplish-blue pigment except where it is dissolved in lipid. Here it appears red. In rat tissues a high aetivity of the enzyme is found in kidney, liver, intestine, testis and epididymis and a lesser activity in salivary glands, pancreas, thymus, traehea, lung, spleen and lymphoid tissue.

Method for β-glucosidase (DW. 160)

Seligman and his co-workers (Cohen *et al.*, 1952b) suggested that the preparation of the β-glucoside of 6-bromo-2-naphthol would enable a histochemical method for the localization of β-glucosidase to be evolved. This substrate can be used in the same manner as in the preceding method for β-galaetosidase, except that fresh sections are essential. Localization is again obtainable on the histological level in every active tissues sueh as eockroach eolleterial glands (Fig. 164, p. 500). The tanning reaction in the oothecae of *Blatta* and *Periplaneta* depends on the liberation of free protocatechuic acid from its β-glucoside, by a β-glueosidase from the right colleterial gland (Brunet and Kent, 1955). Histochemieally it can be shown that both glands possess a β-glucosidase but that on the right side is much more powerful than that on the left. It is possible that the latter exerts a synthetic effect *in vivo*.

Method for α-glucosidase (DW. 159)

An essentially similar method to those recorded above has been used by Rutenburg, Lang, Goldberg, and Rutenburg (1958a) for the demonstration of α-glueosidase in the small intestine and kidney of the rat. In addition to the standard post-coupling technique it was observed that hydrolysis of the substrate was suffieiently rapid for enzyme activity to be demonstrated by a simultaneous eoupling technique.

Method for N-acetyl-β-glucosaminidase (DW. 160)

The occurrenee of N-acetyl-β-glucosaminidase in mammalian tissues was described by Watanake (1936a and b) and the properties of the enzyme have also been recorded by East, Madinaveitia and Todd (1941) who used partially purified preparations from ox liver and testis. The distinetion of this enzyme from hyaluronidase was made by Hahn (1945) and Linker, Meyer and Weissmann (1955) have now shown that the oligosaecharides produced from hyaluronic acid by hyaluronidase are further degraded by β-glucuronidase and N-acetyl-β-glucosaminidase, in the manner illustrated below:

Polysaccharide chain

↓ polysaccharases (Hyaluronidase, Heparinase)

Oligosaccharide fragments

↓ oligosaccharases (Glucosaminidase, Galactosaminidase Glucuronidase)

Sugar Units.

Further studies were made by Pugh, Leaback and Walker (1957) who showed that the enzyme is inhibited by acetate, acetamide and N-acetylglucosamine, all three behaving as typical competitive inhibitors. In 0·05 M-citrate buffer optimum activity was found at pH 4·3.

A histochemical method for glucosaminidase has been developed by Pugh and Walker (1958) which is based on the hydrolysis of α-Naphthyl-N-acetyl-β-D-glucosaminide at pH 4·5 and on the simultaneous capture of the free naphthol by a suitable diazonium salt. Fast Garnet GBC (18) has been found very suitable for this purpose. Details of the method are given in Appendix 17. Localization is histological rather than cytological (Fig. 165, p. 500), since diffusion of enzyme is very difficult to prevent. Cold formalin-fixed tissues are best employed and the incubating medium should contain methyl cellulose or PVP in order to reduce diffusion of lyo-enzyme to a minimum. The final reaction product is lipid soluble and the preparations are therefore not absolutely stable.

Inhibition of glucosaminidase by N-acetyl-glucosamine is relatively ineffective since it has considerably lower affinity for the enzyme than the substrates usually employed. Conchie and Levvy (1957) found that the hydrolysis of simple glycosides was inhibited by the aldonolactone corresponding in configuration to the sugar residue of the substrate and Findlay, Levvy and Marsh (1958) have now shown that N-acetyl-glucosaminolactone (2-acetamido-2-deoxyglucono-lactone) and N-acetylgalactosaminolactone are very powerful competitive inhibitors of the N-acetylglucosaminidase of rat epididymis. Since there may be some overlap in the specificities of the various hexosidases the use of specific inhibitors such as those of the aldonolactone series, should be extended as soon as possible to histochemical studies on the various glycosidases.

The Transglycosylases

The transglycosylases form a large group of enzymes only a few of which are found in animal tissues. They include the phosphorylases

which transfer glycosyl groups to or from phosphate. The best known phosphorylase, glucosan phosphorylase, is known as P-enzyme when it occurs in plant tissues or as amylophosphorylase (Cori *et al.*, 1938; Cori and Cori, 1943). It transfers glucose reversibly from combination with phosphate to the non-reducing end of a polysaccharide chain.

α-D-glucose-1-phosphate + D-glucosyl-R $\rightleftharpoons$ 1,4-α-glucosyl-glucosyl-R + phosphate.

For the reaction to proceed to the right the presence of a polysaccharide starter is necessary, after which it builds up long unbranched chains with 1,4-linkages. This enzyme is demonstrable histochemically.

Another transglycosylase which can be demonstrated by histochemical means is an enzyme which forms branched structures of glycogen and amylopectin from the unbranched chains by transferring part of the chain to the 6-position of one of the glucose residues of the remainder. This enzyme, known as Q-enzyme or " branching factor " is also called amylo-1, 4-1,6-transglycosylase. It is not a phosphorylase, as should be clear from the above description of its activities.

Methods for Glucosan Phosphorylase (DW. 525)

The first histochemical reaction for phosphorylase was that described by Yin and Sun (1947) who demonstrated the presence of the enzyme in plant tissues by catalyzing the production of starch from glucose-1-phosphate. Two years later Cobb (1949) applied the method to fresh sections of cartilage and was able to demonstrate the formation of glycogen in the very active cells of this tissue. In Cobb's procedure pre-existing glycogen was first removed from the sections with amylase and, following incubation, the newly-formed glycogen was rendered visible with an alcoholic PAS reaction. Further development of the method was carried out by Goldberg, Wade and Jones (1952) who used the PAS reaction, Best's carmine, and also the iodine reaction for staining enzymically produced glycogen. As an alternative approach they endeavoured to demonstrate the sites of released phosphate by a modification of Gomori's acid phosphatase reaction, hydrolysis of the glucose-1-phosphate by tissue acid phosphatase being prevented by inclusion of NaF in the medium.

The phosphorylase method has been placed on a sure footing by the studies of Takeuchi and Kuriaki (1955) and Takeuchi, Higoshi and Watanuki (1955) who introduced the use of activators (insulin and adenosine-5-phosphate) and primer (glycogen) without which histochemical performance of the reaction is irregular and unsatisfactory. Takeuchi and his associates tested several methods for staining glycogen formed in the reaction and they concluded that iodine was the most

satisfactory although permanent preparations could not be obtained. The newly-formed glycogen was observed to stain bluish-black with iodine and it was thus easily distinguished from the mahogany-coloured deposits of pre-existing glycogen.

In his second paper (Takeuchi *et al.*, 1955) Takeuchi explained the colour differences as being due to the fact that the newly-formed polysaccharide was of low molecular weight and might be either amylose, amylopectin or perhaps a slightly-branched glycogen. In a third paper (1958) Takeuchi concludes that the phosphorylase method is capable of demonstrating not only phosphorylase but also amylo-1,4-1,6-transglyco-sylase. The same conclusion appears to have been reached independently by Ellis and Montagna (1958). It now seems clear that an unbranched polysaccharide of the amylose type is the reaction product of the phosphorylase reaction and that this stains blue or blue-black with iodine. If the second enzyme is present and active, a branched polysaccharide (glycogen) can be formed subsequently from the amylose when the chain reaches a certain critical length. This is somewhere between 42 and 100 glucose units according to Hassid (1945).

Method for Glucosan Transglycosylase (DW. 526)

Takeuchi has now established a series of tests by which one can distinguish the activity of this enzyme from that of the associated glucosan phosphorylase. Full technical details of the various procedures are given in Appendix 17, p. 898. The chief methods of distinction between the two enzymes are (1) the inclusion of 20 per cent. ethanol in the incubating medium and (2) post-digestion of the reaction products with α- and β-amylase. In the presence of alcohol, activity of the transglycosylase is strongly inhibited. After treatment with α-amylase both amylose and glycogen are digested and the subsequent iodine reaction becomes negative. With β-amylase only amylose disappears and a mahogany stain with iodine then indicates sites of branching enzyme activity. Table 42, below, summarizes Takeuchi's observations on differences between the two enzymes.

TABLE 42

Distinction between glucosan phosphorylase and glucosan transglycosylase.

Reaction	Phosphorylase (Amylose producing)	Transglycosylase (Amylopectin or glycogen)
Iodine	Blue	Mahogany
PAS	Magenta	Magenta
α-Amylase	Complete digestion	Complete digestion
β-Amylase	Complete digestion	Unaltered
Ethanol (20 per cent.)	Moderate Inhibition	Strong Inhibition
Mercuric chloride ($10^{-4}M$)	Moderate Inhibition	Complete Inhibition

Applications of the Two Methods. Most of the applied work in this field has been carried out by Takeuchi and his associates (Takeuchi and Kinoshita, 1956; Sugino, 1957; Watanuki, 1957). Skin phosphorylases, in particular, have been investigated by Braun-Falco (1956) and by Ellis and Montagna (1958), and Carbonell (1955) has investigated the distribution of phosphorylase in the conducting system of the heart. Shimizu and Okada (1957) have given an account of the latter enzyme in developing rodent brain.

The overall distribution of the two enzymes is quite similar in most tissues though there are great differences between one species and another in respect of the presence or absence of the enzymes in a given tissue. The reaction is always cytoplasmic and it is strongest in muscle (Fig. 166, p. 500), nerve and in certain epithelia. In mammalian kidney the strongest activity is found usually in the collecting tubules (rabbit) or in the distal convoluted tubules in the rat (Macpherson and Pearse, 1957; Pearse and Macpherson, 1958). For the most part cells which show strong phosphorylase and transglycosylase activity are those which normally contain glycogen. If utilization is high, however, this relationship may not be observed. In human skin Ellis and Montagna (1958) found high activity of phosphorylase in the stratum basale and of both enzymes in the stratum spinosum. The highest activity was found in children and the lowest in older subjects. Since the epidermis of the common laboratory animals apparently contains neither the phosphorylase nor the transglycosylase it is apparent that their carbohydrate metabolism must be carried out by some alternative pathway.

Widespread application of the phosphorylase methods to normal and pathological tissues can confidently be expected. The reliability of the methods, and the fact that a reasonable interpretation can be put upon changes in level of enzyme, is sufficient to ensure their popularity. Cold microtome sections are essential and these can be used free-floating or mounted on coverslips. Takeuchi himself advises the variation of section thickness depending on the amount of activity estimated to be present. I prefer to use thin sections whenever possible since, especially when ethanolic media are employed, the precision of enzyme localization is such as to warrant their use. The only drawback attached to the use of thin sections is that the result may be patchy. If this occurs it is expedient to incubate a thick section for overall localization and a thin one for intracellular localization.

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CHAPTER XVIII

OXIDASES AND PEROXIDASES

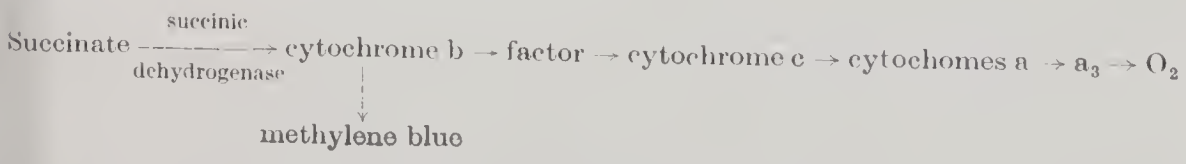
The histochemistry of the oxidases lags behind their biochemistry to a greater extent than it does in the case of the enzymes we have considered in the previous four chapters. Very little critical work has been done with any of the methods described below, in spite of the relative antiquity of some of them. The enzymes we have to consider are *cytochrome oxidase*, *peroxidase*, *catalase*, *phenol oxidase* and D-*amino-acid oxidase*. The first three are hæmoprotein enzymes and the next two are copper proteins and the chapter is therefore divided into three convenient sections, hæmoproteins, copper proteins and others.

The Hæmoprotein Oxidases

Cytochrome Oxidase and the Succinic Oxidase System

The respiratory pigments now known as cytochromes were discovered by MacMunn (1886) but his observations were belittled by Levy (1889) and by Hoppe-Seyler (1890) who maintained that the pigments were hæmochromogens derived from hæmoglobin. After confirming MacMunn's findings Keilin (1925) applied the name cytochromes to the pigments concerned and in a series of papers (Keilin, 1929, 1930; Keilin and Hartree, 1937, 1938, 1939, 1949) he described the nature of the cytochrome-cytochrome oxidase system. An entirely independent series of observations by Warburg (Warburg and Negelein, 1929, 1931) resulted in the description of an iron-containing enzyme called *Atmungsferment* which played an important role in ærobic cellular respiration.

According to the nature of the hæmoprosthetic group the cytochromes are divided into 3 groups, *a*, *b*, and *c*, and these are the main components of the normal ærobic oxidation system of cells. The whole system has been called the succinic oxidase system and it may be written descriptively as follows:



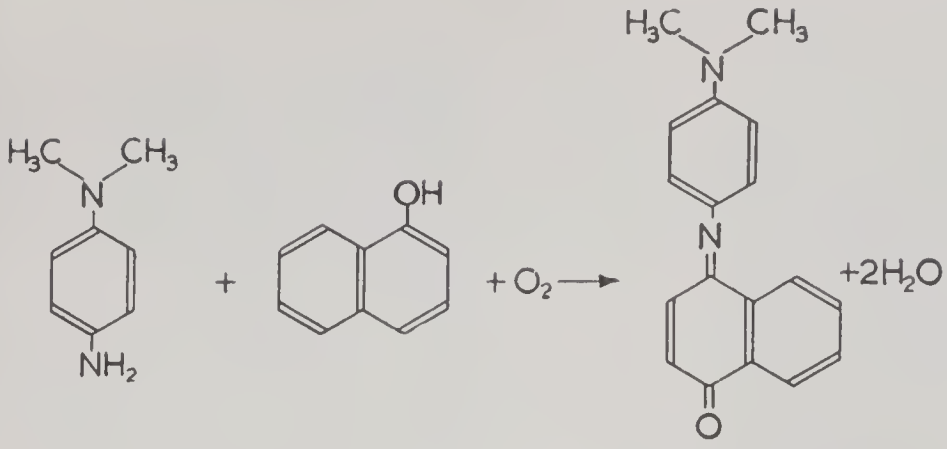
Succinic Oxidase System (Keilin and Hartree)

The proteins to the left of cytochrome c. are collectively known as the succinic dehydrogenase system and those to the right of it as cytochrome oxidase (Singer, Kearney and Massey, 1956), but otherwise, the term cytochrome oxidase is regarded as synonymous with cytochrome a_3 (Keilin and Hartree, 1939; Ball and Cooper, 1952; Smith and Stotz; 1954; Stotz *et al.*, 1956). These last authors have purified cytochrome oxidase and found that it consists of 33·2 per cent. lipid (14·3 per cent. phospholipid). The enzyme is entirely intramitochondrial and it is closely bound to the structure of the mitochondria.

Distribution of the Succinic Oxidase System. The components of this system are distributed widely in the cells of various organisms, often in quantities too small to be demonstrated by histochemical means. The amount of cytochrome oxidase-cytochrome c. which is present in animal tissues depends on the oxidative capacity of the organ concerned. Thus, in the rat, heart muscle and kidney are two of the richest sources. Malignant tumours have often been reported to possess a low cytochrome content (Elliot and Grieg, 1938; Schneider and Potter, 1943a), but this is not always below the normal figure for the tissue of origin. Succinic dehydrogenase is also distributed widely in normal tissues and a number of studies have been made, particularly in the rat, by Meyer and his collaborators (Meyer *et al.*, 1947; McShan *et al.*, 1946; McShan and Meyer, 1946). Since a rise in succinic oxidase activity has been observed in tissues in which the increased respiratory rate is part of a general increase in activity, a large number of studies have been made of malignant and precancerous tissues (e.g. Schneider and Potter, 1943a and b; Slack, 1943 ; Carruthers and Suntzeff, 1947 ; Gallico, 1947 ; Hoch-Ligeti, 1947a and b). Most of these have indicated a gradual increase in one or other of the components of the system with the onset of the malignant change. It is because of these studies that interest in the histochemical demonstration of the succinic oxidase system has increased in recent years.

Inhibitors of Cytochrome Oxidase. The oxidation of reduced cytochrome c. by cytochrome oxidase *in vitro* was found by Keilin (1936) to be inhibited by cyanide or by sodium azide (NaN_3) but *in vivo*, the results were found to be complicated by the presence of other enzyme systems. H_2S also causes inhibition of the enzyme, as does carbon monoxide in the absence of light, by forming complexes with the iron present in the molecule.

Indophenol Oxidase. It has been known for many years that an enzyme was present in the majority of tissues which would catalyze the oxidative reaction between α-naphthol and dimethyl-*p*-phenylenediamine, the so-called " Nadi " reaction, to form indophenol blue. The enzyme responsible was originally known as indophenol oxidase.



Dimethyl-*p*-phenylenediamine | α-naphthol | Indophenol blue

In 1938, however, Keilin and Hartree showed that indophenol oxidase would catalyze the oxidation of reduced cytochrome, and that cytochrome oxidase would produce indophenol blue in the " Nadi " reaction. They concluded as the result of many experiments, that indophenol oxidase, *Atmungsferment*, and cytochrome oxidase were one and the same enzyme. It was therefore evident that the " Nadi " reaction demonstrated the presence of cytochrome oxidase and its substrate, reduced cytochrome c.

We now know, from the work of Keilin and Hartree, that the " Nadi " reaction really proceeds as follows:

$$\alpha\text{-naphthol} + \text{dimethyl-}p\text{-phenylenediamine} + 4 \text{ cytochrome c.} \xrightarrow{\text{spontaneous}} \text{Indophenol blue} + 4 \text{ reduced cytochrome c.}$$

$$\text{And } 4 \text{ reduced cytochrome c.} + O_2 \xrightarrow{\text{cytochrome oxidase}} 4 \text{ cytochrome c.} + H_2O.$$

The α-naphthol of the " Nadi " reagent acts as a mild reducing agent and the cytochrome oxidase part of the succinic oxidase sytem can be demonstrated *in vitro* if the usual reducing (dehydrogenase) system is replaced by any mild reducing agent capable of acting as a hydrogen donor. Examples are *p*-phenylenediamine or hydroquinone. The oxidation of substituted phenylenediamines via the cytochrome system has been studied by Borei and Björklund (1953). These authors found that oxidation of dimethyl-*p*-phenylenediamine by the system was only very slightly increased by the addition of cytochrome c. They therefore considered the possibility that the endogenous cytochrome c. of their preparations, mediating between oxidase and substrate, might not be sufficient to account for the total oxidation observed. It was suggested that the substrate might be directly attacked by the oxidase.

In biological systems the initial oxidation of dimethyl-*p*-phenylene-diamine is rapid but the rate soon falls to zero on account of toxicity of the reaction products. Autoxidation of this compound is rapid, due to metal ion catalysis, and the addition of α-naphthol intensifies this process. The O-R potentials of cytochrome c., and of some of the compounds investigated by Borei and Björklund are given below:

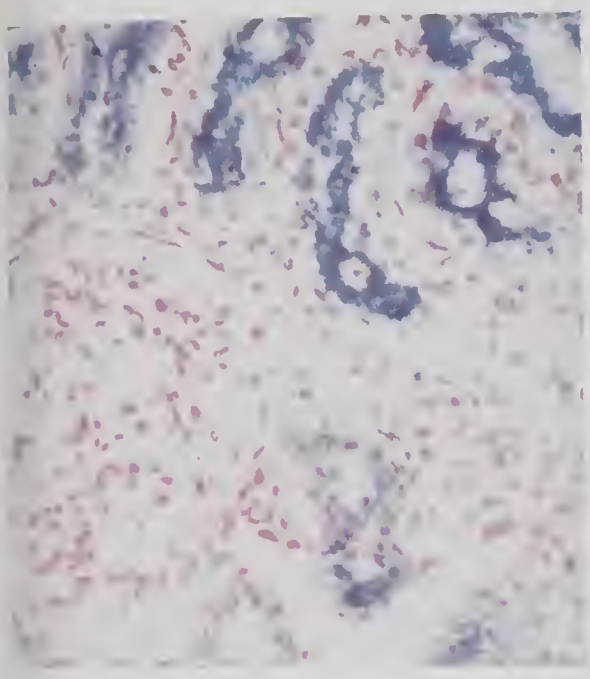
	$E°_{pH7}$
Cytochrome c.	+ 0·253 v.
Quinol	+ 0·273 v.
p-Aminophenol	+ 0·319 v.
p-Phenylene diamine	+ 0·387 v.
Dimethyl-*p*-phenylene diamine	+ 0·335 v.
Tetra methyl-*p*-phenylene diamine	+ 0·222 v.

The O-R potential of cytochrome c. is thus seen to be lower than any of the usual reductants but this does not prevent cytochrome c. from acting as an effective carrier in oxidizing various compounds since a substance of higher O-R potential can continue to reduce one of lower potential if the oxidized state of the former is unstable.

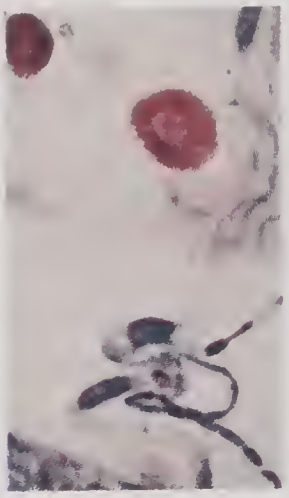
The Cytochrome-Oxidase Reaction (DW. 366)

Historical Survey

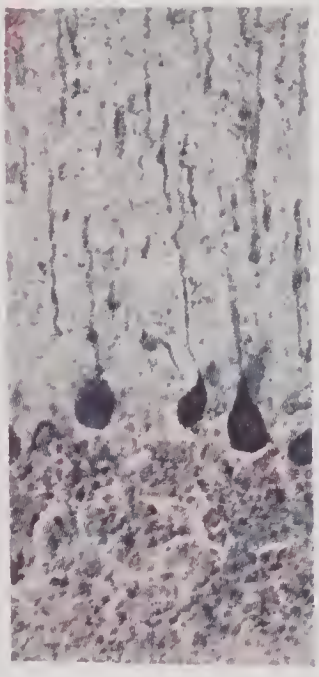
Two methods were given by Lison (1936) under the heading of "Nadi-oxidase" reactions, and both were described as indicating the presence of phenoloxidases. The first, stable, or M-Nadi oxidase reaction was evolved by Schultze (1909a and b) and performed on formalin-fixed frozen sections, while the second, labile, or G-Nadi oxidase reaction (Gräff, 1916) was performed on fresh tissues. The first method was largely employed in hæmatological research since the granules of cells of the myeloid series were found to be invariably positive, as well as the granules of serous salivary glands and lachrymal glands in human tissues. Permanent preparations could be obtained by treating the blue granules with Lugol's iodine, turning them brown in the process, and mounting in an aqueous medium. The M-Nadi oxidase method cannot be considered as an indicator of the cytochrome oxidase-cytochrome c. complex, since this is totally inactivated by formalin. It was at one time considered to be due to the presence of a monophenol oxidase since with this group of enzymes some activity remains after treatment with formalin (see tyrosinase methods, below). More recent work, described later in this chapter, attributes M-Nadi oxidase activity to peroxidase. A reaction of similar type was described by Loele (1912, 1913) using formalin-fixed material and an alkaline solution of α-naphthol. Bluish-violet granules were produced in certain



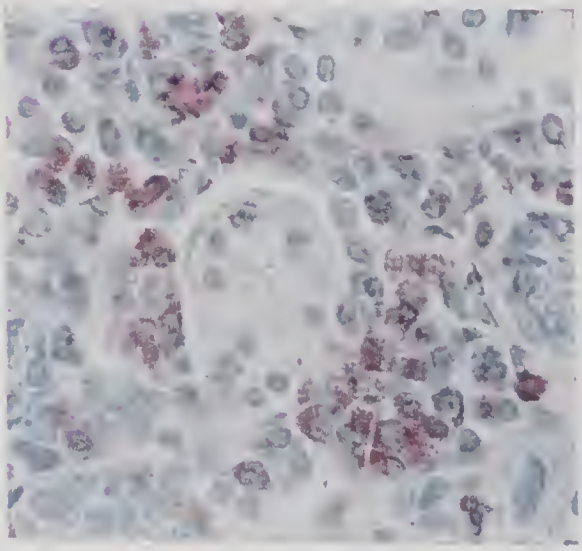
VIA. Rat kidney (10 μ frozen section). β-glucuronidase in proximal (above) and distal (lower right) convoluted tubules. Modified Friedenwald and Becker method. × 270.



VIB. Rat epididymis. Intense activity of β-glucuronidase in fibroblasts and in a smooth muscle fibre. Fishman-Baker modification — Neutral red. × 400.



VIC. Rat cerebellum. Intense β-glucuronidase activity in cytoplasm and processes of the Purkinje cells. Moderate activity in the granular layer. Fishman-Baker modification. Neutral red. × 200.



VID. Human kidney. Case of myeloid leukaemia. All the myeloid cells contain bright red thioindigo, indicating presence of an "oxidase" system. 4 - Methyl - 6 - chlorothionaphthene-Haemalum. × 420.

[*To face p.* 512.

cells, notably those of the myeloid leucocyte series, and the enzyme responsible was characterized as an α-naphthol oxidase. Alternatively it may be due solely to the presence of fatty peroxides of the type shown by the Dam reaction (Chapter XXIII). Neither the M-Nadi oxidase reaction nor the Loele method are of use in diagnostic enzyme histochemistry.

Of greater significance is the second (G-Nadi oxidase) method, which certainly indicates the presence of cytoehrome oxidase-eytochrome c. With this method Gräff showed that the pH of the ineubating medium was of great importance, and that the optimum value varied with the type of cell under consideration. He observed that with animal tissues the optimum pH was between 7·8 and 8·2, while in the case of plant tissues it was between 3·4 and 5·9. Objections to the specificity of the " Nadi " reaction in histochemistry are many and varied. It has been suggested that since indophenol blue is formed from the initial reagents in the presence of molecular oxygen alone, the reaction cannot be specific. Oxidation by air occurs very slowly, however, and this cannot be a significant factor. A more reasonable objection is that indophenol blue can be used as a stain for lipids, and a certain similarity between the results obtained with the usual lipid stains and those produced by the " Nadi " reaction was early observed. Not all lipid inclusions are shown by the latter method, however, and Lison maintained that whereas the " true reaetion " gave a strong blue colour, the " false reaction " of lipids was pale and of a lilac hue. Hollande (1924), however, showed that the identical appearances of the " Nadi " reaction could be obtained by using indophenol blue as a stain. He believed that the granular localization of the oxidase reaction was secondary and that it represented staining of the granules by the dye produced in the cytoplasm of the cell. The granules, in fact, were not oxidase-positive but *indophenophile*. As a corollary, Hollande suggested that in the absence of granules the reaction remained diffuse and invisible under the microscope. Prenant (1924, 1925) was able to reproduce Holland's findings but he disagreed with that author's interpretations, maintaining that the oxidases were not diffuse but attached to the granulations. More modern work agrees substantially with Prenant's views in locating the succinic oxidase systems predominantly in particles of the size and character of mitochondria (Hogeboom, Schneider and Palade, 1948). Indophenol blue is insoluble in water so that diffusion away from the site of production, other than possibly into lipid substances, might be expected to be small.

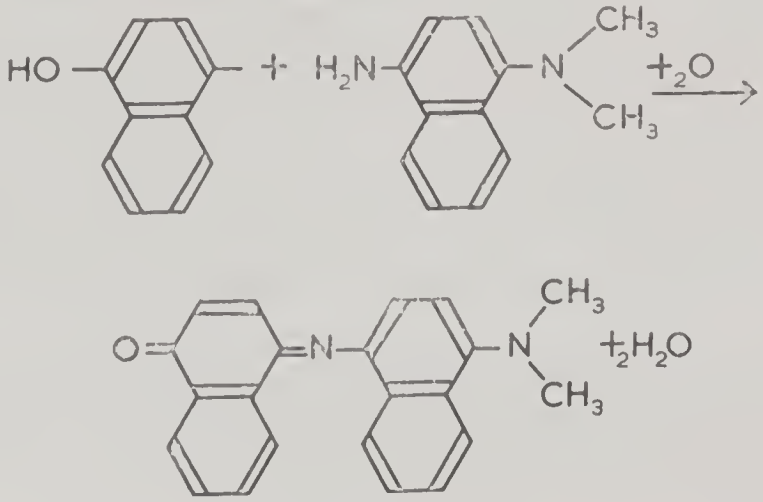
No attempts were made to check the speeificity of the histochemical " Nadi " reaction for cytochrome oxidase, by the use of specific inhibitors, until the work of Moog (1943). It is known that the succinic

oxidase system is instantaneously and reversibly inhibited by cyanides, sodium azide or hydrogen sulphide, on account of their combination with the trivalent Fe of cytochrome oxidase. Moog used 0·005M-sodium azide, treating fresh tissues with this reagent in saline at pH 5·8 for a short period and then transferring the material to freshly prepared "Nadi" reagent containing 0·005M-azide. Inhibition was complete. Using the "Nadi" reaction without any inhibitor Moog found that identical results were obtained at pH 5·8 and at pH 7·2. Since the coloured oxidation products of both the "Nadi" reagent and benzidine are easily reduced to colourless substances if the reducing systems of the cell are intact, it is not always clear that a negative result is really due to lack of oxidase. If the reducing systems are blocked, or otherwise inactivated, a negative result more clearly indicates lack of oxidase and Moog therefore incubated control sections in the presence of phenylurethane which would block the reducing systems. She observed no difference between these sections and those incubated in the absence of phenylurethane. Modifications of the reaction were also made by Perner (1952) who described the deposition of indophenol blue in onion skin cells as microsomal. Numerous attempts to make the reaction permanent were all more or less unsuccessful.

The reagents used by Moog, and their method of preparation, are given in Appendix 18, although the demonstration of cytochrome oxidase by means of the original "Nadi" reagents has now been superseded.

Modern Histochemistry of the Nadi Reaction

The recent studies of Nachlas, Crawford, Goldstein and Seligman (1958) constitute a very considerable advance in the histochemistry of cytochrome oxidase. These authors have replaced the dimethyl-*p*-phenylenediamine of the original reaction with a new compound 4-amino-N,N-dimethylnaphthylamine (ADN). The new "Nadi" reaction, shown in the equation below, gives rise to an insoluble pigment which the authors call indonaphthol purple.



A number of naphthols were tested as substitutes for α-naphthol but none were satisfactory from the histochemical point of view. Both the rate of autoxidation, and of oxidation catalysed by added cytochrome c., are observed to be slower with the new reagent than with the original "Nadi" reagents but the overall histochemical result (Fig. 167, p. 520) is far more satisfactory. From this point of view the properties of indonaphthol purple are much better than those of indophenol blue. In particular, crystallization is no longer observed and the pigment is stable for a considerable period. Post-fixation of the preparations in formalin must be avoided, however, since this reagent causes definite fading of the pigment. Indonaphthol purple has two outstanding deficiencies; it is not substantive for protein, and it remains lipid soluble like indophenol blue. It is deposited in the tissues in the form of small granules and these appear to be intramitochondrial (Fig. 168, p. 520) especially when protective media have been employed. The granules have a tendency to coalesce during storage and it is advisable to photograph the fresh preparations if a permanent record is required of the finer localizations of eytochrome oxidase. Full details of the new method, and for the synthesis of ADN, appear in Appendix 18, p. 900.

A similar improved cytochrome oxidase method has been reported by Burstone (1959). This author has tested a number of amines in place of the classical one, of which he finds N-phenyl-*p*-phenylenediamine to be generally the most useful. As couplers he has used not only α-naphthol and a variety of substituted naphthols (e.g. 1-hydroxy-2-naphthoic acid ; 1-hydroxy-2-acetonaphthone), but also a series of reactive methylene compounds. The most useful of these appear to be 1-phenyl-3-(*m*-nitrobenzamidopyrazolone) and α-α′-terephthaloylbis (5-chloro-2, 4-dimethoxyacetanilide). The results (Fig. 169, p. 520) are excellent and permanent preparations can be obtained by post-chelation with a variety of metals, cobalt being the best of these. Details are given in Appendix 18, p. 901.

Effects of pH and added Cytochrome C.

Although cytochrome oxidase is active over a wide range of pH most workers have performed the histochemical reaction at pH 7–8. Moog (1943), however, used pH 5·8 in her studies on chick embryos. Nachlas *et al.* (1958) found that maximal pigment production occurred between pH 7·2 and pH 7·4 although between pH 6·6 and 7·6 the differences observed were never very great.

Addition of cytochrome c. to the incubating medium usually increased the rate of pigment production but the routine use of added cytochrome c. was not found necessary or advisable.

Inhibitors. The indonaphthol purple reaction for cytochrome oxidase is eompletely inhibited by KCN (10^{-3}M). No other control is necessary. Nachlas *et al.* observed that reduction of the pigment to a colourless product by endogenous dehydrogenase did not occur and they found no increase of pigment production by using urethane (cf. Moog, 1943) to block activity of eytochrome b. On the contrary, inhibition of cytochrome oxidase by this compound was noted.

Alternative Methods for Cytochrome Oxidase

Observing that colorimetric assay of cytochrome oxidase activity could be made *in vitro* by the use of redox dyes Crawford and Nachlas (1958) attempted to produce a histochemical reaction based on this principle. They tested seven redox dyes, in reduced or leuco form, but in no case did the oxidation product remain at the site of enzyme activity.

The Biochemistry of the Peroxidases

There are three enzyme systems which destroy H_2O_2 in the tissues, cytochrome c.-peroxidase, catalase and the various peroxidases. The first, which was described by Altschul, Abrams and Hogness (1940), can oxidize the reduced form of cytochrome c. only at the expense of peroxide. It cannot, therefore, be demonstrated histochemically by methods making use of a chromogenic substrate for oxidation. Catalase, which also occurs in both animal and plant tissues, splits H_2O_2 into water and molecular oxygen. Like cytochrome c.-peroxidase, it cannot be shown histochemically with the usual chromogenic substrates since H_2O_2 acts both as substrate and acceptor. The peroxidases, which can be demonstrated by means of chromogenic substrates, are weak or lacking in most animal tissues though they occur in moderate amounts in mammary gland and in the granules of leucocytes of the myeloid series. The mammalian peroxidases differ from those occurring in plants in that the hæm compound they contain is green in colour, and they are strictly classified as verdoperoxidases. Most hæm and hæmatin compounds exhibit weak peroxidase activity, a property made use of histochemically for the demonstration of hæmoglobin in the red blood cells (see below). Peroxidases catalyse reactions of the type $AH_2 + H_2O_2 \rightarrow A + 2H_2O$, and the peroxide-peroxidase system thus causes the rapid oxidation of substances which peroxide alone oxidizes extremely slowly. The need for the presence of H_2O_2 by peroxidases was made use of by Agner (1941), who demonstrated that the "Nadi" reaetion of leucocyte granules could be abolished by the addition of eatalase, indicating that their "Nadi oxidase" was, in fact, a peroxidase.

The present status of the peroxidases has been admirably set forth in a recent review by Saunders (1957). Three enzymes in this class have now been obtained in crystalline form. They are verdoperoxidase (leucocyte), lactoperoxidase (milk) and horseradish peroxidase. Most biochemical work has been done with the latter. The peroxidase system is capable of oxidizing a variety of amines to coloured compounds and it will also oxidize phenolic methyl groups via aldehyde to quinone (Booth and Saunders, 1950). Hughes and Saunders (1954) have shown, furthermore, that the system is capable of breaking carbon-halogen bonds, including the C-F bond. During the oxidation of *p*-iodoaniline the iodine atom is eliminated and then oxidized to I_2, which can then iodinate a suitable acceptor. This process of transiodination is of considerable importance in relation to the iodination of tyrosine compounds in thyroid metabolism (see below).

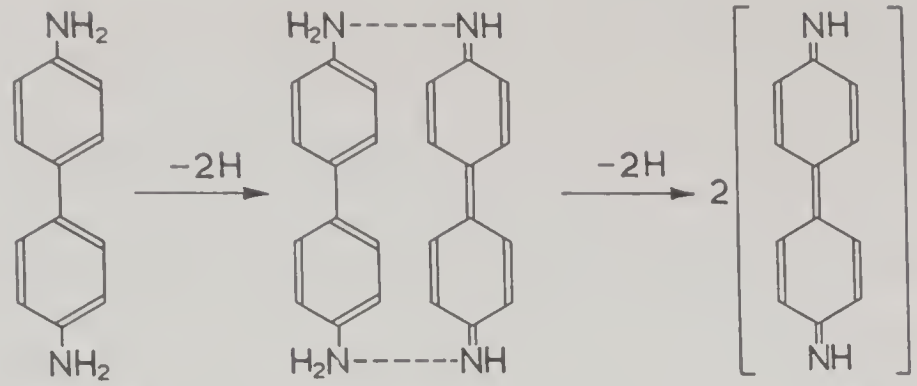
Interest in the peroxidases has mainly been confined to the study of the leucocyte granules and this has been the province of the hæmatologist. In recent years, however, a more general interest has been revived by the finding of peroxidase activity in the thyroid acini and colloid by Dempsey (1944) confirmed by de Robertis and Grasso (1946). Since peroxidase catalyses the liberation of iodine from inorganic iodides by hydrogen peroxide, its presence in the thyroid would serve a useful function. Both Dempsey, and the other authors quoted, observed that the reaction was inhibited by low concentrations of thiourea and that much larger concentrations of thiourea were required to inhibit the peroxidase activity of red blood cells. Glock (1944) criticized the specificity of Dempsey's findings on the ground that the concentration of thiourea necessary to inhibit peroxidase activity in the horse thyroid was of the order which inhibited red cell activity, and Randall (1946) showed that thiols did not inhibit peroxidase at all, but simply reduced the coloured products formed by the peroxidase-catalysed reactions.

Histochemical Methods for Peroxidases (DW. 381)

Few of the many oxidizable substrates for the peroxidase system give rise to products sufficiently deeply coloured to be visible in tissue sections under the microscope. Three substances or groups of substances, in particular, have been used in histochemistry ; they are benzidine, α-naphthol and various leuco-dyes.

Benzidine Reactions

These reactions depend on the oxidation of benzidine by the peroxide-peroxidase system to a blue or brown product. The formula reproduced below was given by Lison (1936, p. 166).



The intermediate product, benzidine blue, is unstable and is oxidized to the brown compound without enzymic intervention. The reaction can take place over a wide pH range and no attempt is usually made to control the pH by means of buffers. Some authors recommend acidification of the benzidine-peroxide medium and state that if an acid solution is used the resulting colour is predominantly blue rather than brown. If the explanation given by Lison is correct this indicates that the stability of the intermediate compound is increased. Some authors (Garcia Blanco and Grisolia, 1946) make a histochemical distinction between pseudoperoxidase and true peroxidase reactions, separating the red cell from other cytoplasmic peroxidases ; others make a similar distinction by treatment with heat, since the pseudoperoxidases are relatively heat stable. Although it may be necessary, as in the case of the thyroid acinar cells for instance, to establish that the positive reaction is not due to hæmoglobin, the peroxidase of the latter should not be distinguished as pseudoperoxidase.

The optimal concentration of H_2O_2 for the peroxidase reaction is about 0·05M (1 drop of 10 vol. H_2O_2 per ml.) and higher concentrations may inhibit the reaction by destroying the enzyme. Certain substances have been found to intensify the reaction and Mancini and Villamil (1950) describe the action of molybdate in this respect as that of a catalyser. The exact meaning of the term, in this context, is uncertain. Ettori (1949), using *in vitro* methods for the estimation of peroxidase, has shown that at pH 5·9, the rate of enzyme activity is increased by increasing the concentration of phosphate buffer up to an optimum level of 0·07M. In the presence of phosphate buffer the harmful effect of excess H_2O_2 is considerably reduced. Improvements in the technique of the benzidine-peroxidase reaction have been reported by Mitsui and Ikeda (1951) and by van Duijn (1955). These improvements are based on the observation that if a high salt concentration is maintained the blue intermediate compound (above) precipitates in the form of stable crystals. Suitable conditions were established by Mitsui and Ikeda with nickel ammonium sulphate and by van Duijn

with the addition of 5 per cent. ammonium chloride to the incubating medium. The Japanese authors reported that their mixture was stable for months ; van Duijn's must be freshly prepared since after 30 minutes benzidine blue begins to appear spontaneously. This can be controlled to some extent by addition of EDTA. The improved technique of van Duijn is given in Appendix 18, p. 903.

TABLE 43

List of Benzidine-peroxide Methods

Author	Material	Fixative or Mordant
Graham (1918)	Smears	Formalin-alcohol
Goodpasture (1919)	Smears and sections	Formalin (optional)
McJunkin (1922)	Paraffin sections	Formalin
Prenant (1924)	Fresh tissues	None
Sato *et al.* (1925)	Smears	Copper sulphate
Loele (1927)	Smears	Various
Washburn (1928)	Smears	None
Armitage (1939)	Smears	Formalin-alcohol
Mancini and Celani (1940)	Smears and sections	Ammonium molybdate
de Robertis and Grasso (1946)	Frozen sections	Ammonium molybdate
Cazal and Roure (1947)	Smears	Formalin-alcohol-$CuSO_4$
Villamil and Mancini (1947)	Frozen sections	Ammonium molybdate
Bonati and Franchi (1948)	Smears	None
van Duijn (1955)	Frozen sections	None

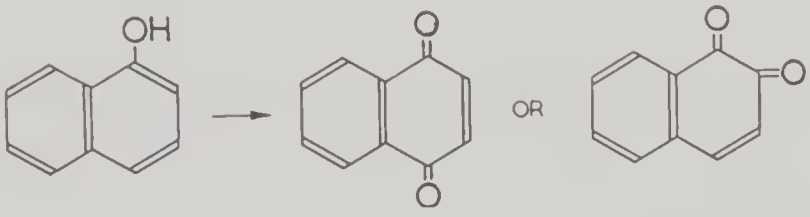
Methods for air-dried fixed or unfixed smears of blood and bone marrow are dealt with in text books of hæmatology, and some of them appear in Table 43, which is a list of the various benzidine-peroxide methods, applicable both to smears and to sections. The only methods for which technical details are given in Appendix 18 are the methods of de Robertis and Grasso (1946), and of van Duijn (1955). These, and especially the second, are recommended for all histochemical investigations into the peroxidase activity of normal or pathological tissues.

Benzidine Substitutes. The possibility of using alternatives to benzidine seems to have been little explored in histochemical practice. On account of its carcinogenic qualities this compound has been replaced by *o*-tolidine in tests for blood in urine or fæces, and *o*-tolidine can be used in place of benzidine as a reagent for the demonstration of copper-containing pigments in the marine invertebrates (Chapter XXIV, p. 698). Another alternative is mesidine, the chemistry of whose oxidation by a peroxide-peroxidase system was explored by Chapman and Saunders (1941). The oxidation product, 2,6-dimethylbenzoquinone-4-(2′,4′,6′-trimethylanil), is purple in colour and apparently poorly soluble in water. Mesidine has not been used in histochemical investigations of the peroxidases. The use of thioindoxyls

(3-hydroxythionaphthenes) as hydrogen donors in oxidase systems, introduced by Pearse (1954), has not been fully explored. The halogen-substituted thioindoxyls give rise to brightly coloured, insoluble and stable pigments on oxidation which compare favourably with the reaction products of most oxidase reactions reported in this chapter. Thioindoxyls are oxidized by the granules of mammalian eosinophils and by cells of the myelocyte series, especially those present in certain types of leukæmia. The reaction in cells from a case of myeloid leukæmia (chloroma) is shown in Plate VIb, p. 512. It takes place between pH 6·0 and 9·2 and between 20° and 65° and, in formalin-fixed tissues, proceeds without the addition of H_2O_2. It has been postulated that the reaction is due to a peroxide-peroxidase complex which can be inhibited by ferricyanide, catalase and by excess H_2O_2. A possible source of peroxide in formalin-fixed tissues, which might be responsible for the formation of the complex, is methyl hydroperoxide ($CH_3.O.OH$). This is present in most commercial samples of formalin.

The α-Naphthol Reactions

α-Naphthol has been used less often than benzidine for histochemical peroxidase reactions. It gives a red colour on oxidation, probably due to the formation of quinones.



The two principal methods using this substance are those of Graham (1916) and of Ritter and Oleson (1947). The former was designed for use on fresh unfixed blood smears and the latter, which is given in Appendix 18, employs thin blocks of unfixed tissue which are subsequently fixed and embedded in paraffin. It is not very satisfactory, in practice, and the benzidine methods are to be preferred. It is probable, in fact, that the α-naphthol methods demonstrate only the so-called stable sudanophilia of Lillie and Burtner (1953).

The specificity of the α-naphthol reaction is in any case doubtful, as shown by Gomori (1953), since a reaction can be obtained in the absence of added H_2O_2. The possibility that fatty peroxides might take part in the reaction is made unlikely by the fact that if the reaction product is removed the reaction can be repeated. Using horse eosinophil leucocytes Vercauteren and Blondé (1954) were unable to demonstrate the participation of fatty peroxides in the α-naphthol or benzidine reactions. On the whole it seems that the α-naphthol reaction

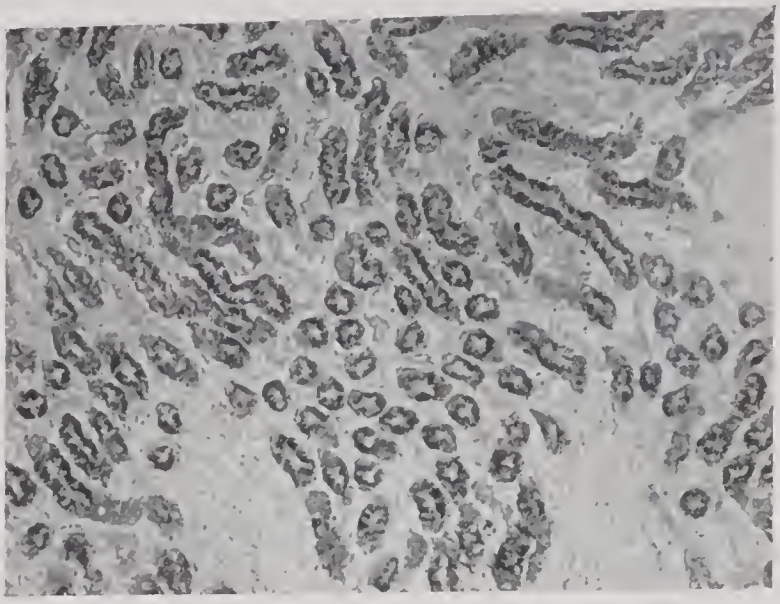


FIG. 167. Rat kidney. Cytochrome oxidase activity throughout the cytoplasm of the tubular cells. ADN method. × 42.

FIG. 168. As Fig. 167. High power view of single tubule. Cytochrome oxidase appears in the form of (intramitoehondrial) dots. ADN method. × 2,224.

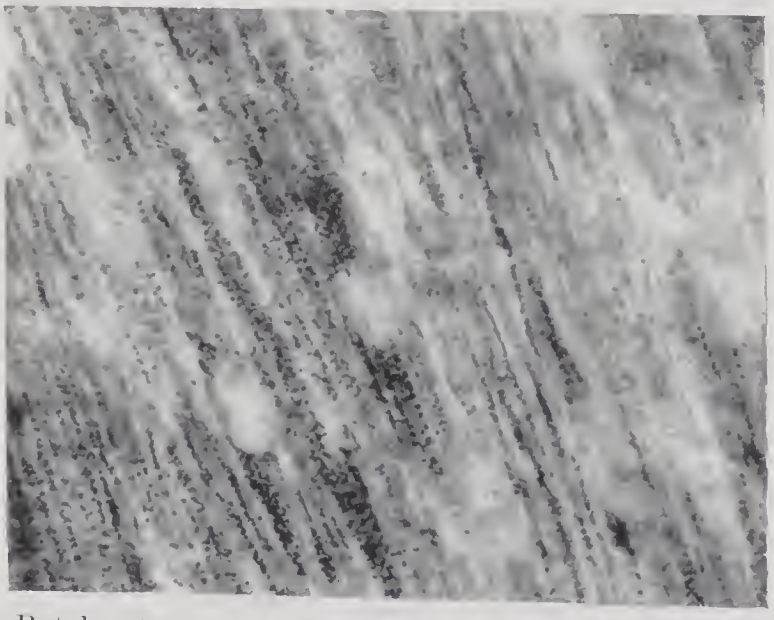


FIG. 169. Rat heart muscle. Granular distribution of cytochrome oxidase in sarcosomes. Burstone's method. × 775.

[*To face p.* 520.

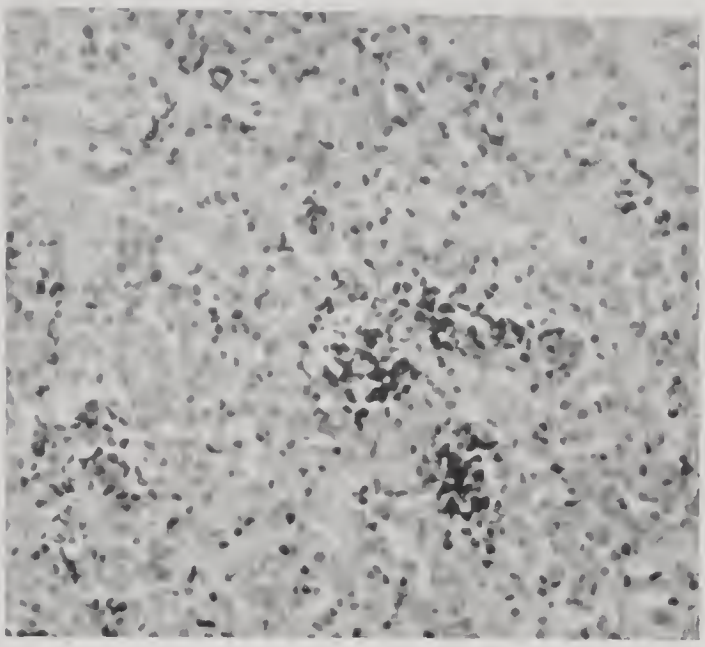


FIG. 170. Human spleen stained by the leuco-Patent Blue method for hæmoglobin. × 190.

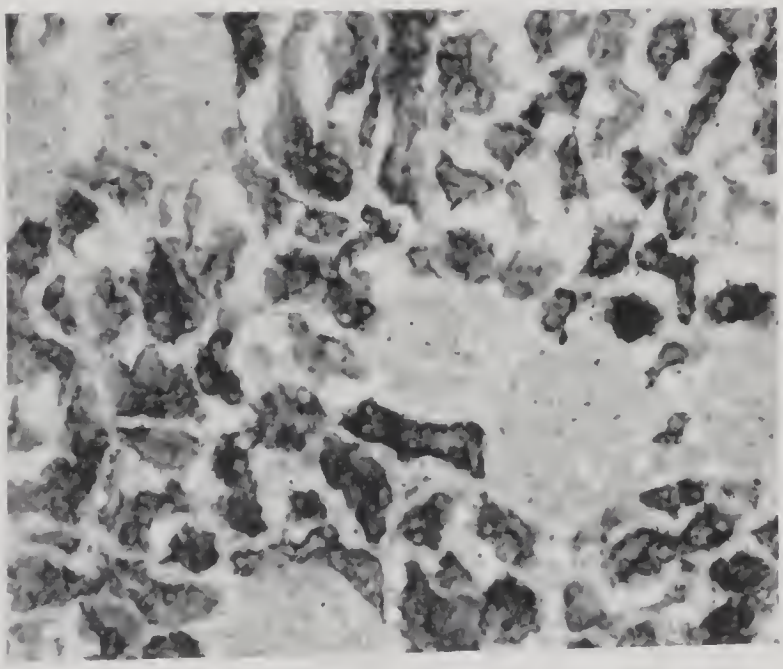


FIG. 172. Rat kidney. Distribution of monoamine oxidase by naphthoic acid hydrazide method. × 136.

is not enzymic in nature and that it depends on the presence of undetermined oxidation products in solutions of α-naphthol.

Leuco-dye Reactions

Lison (1936) introduced the use of acid fuchsin and other dyes, reduced by means of nascent hydrogen, as indicators for the peroxide-peroxidase systems. These zinc-leucos, as he called them, were produced by boiling the dye with powdered zinc and acetic acid until the colour of the original solution was discharged. Although Lison suggested that preparations made by the above methods were both beautiful and stable, they have not been much used by other workers. Fautrez and Lambert (1937) published a method for the demonstration of hæmoglobin in fixed tissue sections using leuco-Cyanol (xylene-Cyanol FF), but they did not apparently consider that the method could be used as an indicator of peroxidase activity. Lison (1938) introduced a leuco-Patent Blue technique for the identification of hæmoglobin casts and globules in the kidneys of frogs injected with hæmoglobin solutions. He used a formalin-ferricyanide fixative and frozen sections. Patent Blue is a triphenylmethane dye which closely resembles Cyanol except that it possesses four instead of two ethyl groups and no methyl group. Dunn and Thompson (1946) modified Lison's Patent Blue method for use on paraffin sections of material fixed in neutral formalin and considered the reaction to be of the "peroxidase type." Dunn (1946) described an essentially similar method using leuco-Cyanol and stated that its specificity for hæmoglobin was equal to that of the Patent-Blue method. Since both these are essentially peroxidase reactions the granules of certain leucocytes, especially the eosinophils, stain blue by either. Fig. 170 shows red cells in splenic sinusoids demonstrated by the leuco-Patent Blue method, using formalin fixed tissue.

Histochemical Method for Catalase (DW. 383)

Many unsuccessful attempts have been made to produce a histochemical reaction for catalase. Only one report of a successful attempt has so far appeared in the literature (Nishiyama and Kobayashi, 1953). Their method is based on the observation by Stern (1936) that acetaldehyde is produced by the action of catalase on monoethyl hydrogen peroxide. The authors synthesized this substrate by the method of Rieche (1929) and used it in a histochemical procedure with Schiff's reagent as the final demonstrator of the aldehyde. Inhibition by hydroxylamine and by KCN was considered to indicate the specificity of the method but the former, at least, would be more likely to exert its effect by blocking pre-existing aldehydes. Some type of aldehyde

blocking procedure is an essential prerequisite of methods of this type. The reaction was thought to take place as follows:

$$C_2H_5OOH \xrightarrow{\text{Catalase}} CH_3CHO + H_2O$$

$$+$$

Schiff's Reagent

$$\downarrow$$

Red Product

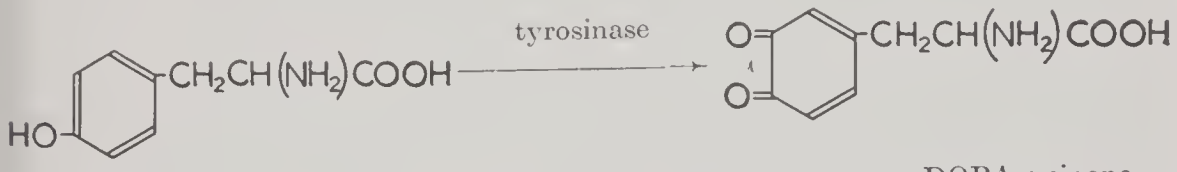
There are so many basic objections to the above principle that the use of the method, as it stands, cannot be advised. The primary reaction product is freely soluble and no evidence has been produced to show that the final red dye is not soluble also. The introduction of an effective capture reagent is absolutely necessary and the obvious reagent for this purpose is 2-hydroxy-3-napthhthoic acid hydrazide. The method is given in Appendix 18 so that those who feel disposed to attempt the necessary modifications can proceed without hindrance. A mechanism of this type has in fact been suggested by Watanabe (1958) as the basis of a method for " hydroperoxidase." This author regards the histochemical separation of catalase and peroxidase as unrealistic.

Copper-Protein Oxidases

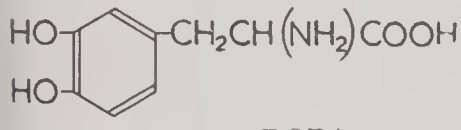
The Biochemistry of Tyrosinase (DOPA Oxidase)

The enzyme tyrosinase, which is widely distributed in plants and animals, is responsible for the conversion of tyrosine into melanin. Principally on account of the histochemical work of Bloch (1917), the precursor of melanin in animal tissues was considered to be dihydroxyphenylalanine (DOPA) and the enzyme concerned with its oxidation was called DOPA-oxidase. In the decade following Bloch's publication of his original method various objections to its specificity were put forward. Przibram (1922) considered that DOPA might be transformed into melanin by two processes, first, by a simple oxidation in alkaline solution, and secondly, by enzyme action at a neutral pH. He disagreed with Bloch's contention that DOPA-oxidase did not act on tyrosine but agreed that the cells demonstrated by the DOPA reaction contained an enzyme of some sort. Hasebrock (1921) investigated the cause of pigmentation in moths and showed that in the egg or larval stage only DOPA could be oxidized to a black pigment, but that in the chrysalis or in the adult moth both DOPA and tyrosine could be so dealt with. These views are substantiated by the findings of Lerner *et al.* (1949) that tyrosinase causes only slow oxidation of tyrosine unless a small amount of DOPA is present, when the speed

of oxidation approaches that of DOPA itself. Although the majority of authors agreed that enzyme action was responsible for the production of pigment from DOPA, considerable doubt was felt in identifying DOPA-oxidase with the enzyme of normal melanin production, since its substrate could not be demonstrated in mammalian tissues. The only difference between tyrosine and DOPA is the presence of a second hydroxyl group on the benzene ring and it is now considered that the oxidation of tyrosine proceeds in the following manner. It is easy to see that the effect of the second hydroxyl group of DOPA is to facilitate oxidation at that locus.

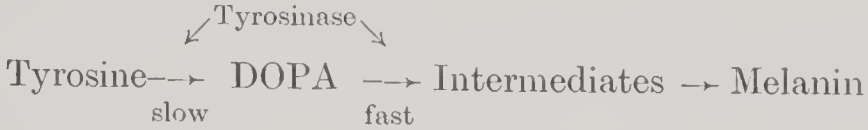


Tyrosine DOPA quinone



DOPA

If the views of Raper (1926) and of Evans and Raper (1937) are correct, DOPA is to be regarded as the first stage of the oxidation of tyrosine by tyrosinase. These views were based on work on the plant enzyme and they cannot necessarily be applied to animal tissues. Hogeboom and Adams (1942) first showed that tyrosinase as well as DOPA-oxidase could be demonstrated in mouse melanoma and Lerner *et al.* (1949) established the identity of these two enzymes in the same mammalian tissue (Harding-Passey mouse melanoma). These authors were not able to separate tyrosinase and DOPA-oxidase activity, although it was possible to prepare fractions which oxidized tyrosine only very slowly. They concluded that a single enzyme or enzyme-complex was involved in the oxidation of both substrates to melanin and this view is illustrated diagrammatically below.



In view of this evidence it is convenient to regard the enzyme demonstrated by the DOPA reaction as " tyrosinase " and to assume that it is capable of acting either as a monophenol oxidase or as an *o*-diphenol oxidase. According to Kertész (1952) the so-called tyrosinase is really a very complicated system composed of (1) an enzyme with copper as its prosthetic group, specific for *o*-diphenols ; (2) an *o*-diphenol and

(3) certain free metallic ions. The complexity of this system is increased by the interdependence of its components since the quantity of *o*-diphenol (or *o*-quinone) depends on the amount of enzyme and on the quality and quantity of the metallic ions present. Kertész considers that the only justification for retention of the term "tyrosinase" is an historical one and it is retained in this section for that reason.

Histochemical Reactions for DOPA-oxidase (DW. 369)

The original method of Bloch (1917) employed agar-embedded fresh tissues from which thick frozen sections were cut. These were incubated with a freshly prepared dilute aqueous solution of DOPA at 22° or 37° for 6–24 hours. Laidlaw (1932) and Laidlaw and Blackberg (1932) noted that the substrate solution need not be fresh but could be stored at 4° and used until a red colour developed. These authors modified the original method by stabilizing the pH of the substrate solution at pH 7·4 with phosphate buffer. Until this time no particular notice had been taken of the effect of pH on the reaction, but Sharlet *et al.* (1942) noted that raising the pH increased and lowering the pH decreased the rate of the reaction ; Lerner *et al.* (1949) emphasized, as was already known, that at an alkaline pH DOPA is oxidized in the presence of oxygen without the need for a catalyst. Possibly a pH of 6·8, which these authors employed for their *in vitro* estimations, should be the highest used in the histochemical DOPA reaction. Becker, Praver and Thatcher (1935) introduced a modified paraffin DOPA technique which has many advantages over other methods and is reliable and easy to use. The details given in Appendix 18, p. 905, are those of a modification applicable to formalin-fixed frozen sections.

Laidlaw cautioned against leaving sections in the incubating medium after the latter had become brown. Becker, on the other hand, recommended that both solution and block should be brownish-black at the end of incubation. A positive DOPA reaction consists of darkening of the entire protoplasm of the cells (melanoblasts in the case of skin) which contain the enzyme. Preformed melanin retains its original colour. Becker considered that only a positive reaction was significant and that with a negative one it might be impossible to determine whether the reaction was really negative or whether it had failed to occur because of some undetermined factor in the technique. If this is true, the usefulness of the reaction in applied histochemistry is diminished, but I consider his view to be unnecessarily cautious.

The degree of accuracy of localization achieved by the method can still be criticized and, as with all enzyme techniques, we must question whether diffusion of the enzyme or of the reaction products, or both,

occurs. A proportion of the enzyme is contained in intracellular cytoplasmic granules from which it might be expected not to diffuse easily and the insolubility of the final product (melanin) cannot be questioned. The intermediate oxidation products such as DOPA-quinone, however, may well be sufficiently soluble to diffuse from their site of production. In practice it is found that with unfixed material some diffusion does occur and this is prevented by short (4 hours) fixation in formalin. Thus the diffusion effect may be due either to diffusion of the enzyme or to diffusion of protein-bound intermediate reaction products, since both these might be expected to be abolished by a protein fixative. Diffusion of the free intermediate reaction product can still occur with fixed tissues and for this the explanation may be that it is no longer bound to proteins in the immediate vicinity of its production, due to blockage of their binding groups by formalin.

The histochemical interpretation of the DOPA reaction was critically reviewed by van Duijn (1953, 1957a and b) who carried out a number of experiments to test its nature. He concluded that many of Bloch's so-called inactivations were due to loss of a soluble factor from unfixed sections. The effect of KCN was also fully considered by van Duijn who pointed out that in their original report Bloch and Schaaf (1925) stated that pretreatment with 0·005 M-KCN, buffered to neutrality, allowed a maximal DOPA reaction to occur. This statement has been repeatedly misquoted in the literature. In his experiments van Duijn found that concentrations of cyanide higher than 0·008 M inactivated the system. He concluded (1953) that the DOPA factor could not be related to any known enzyme and his later studies (1957a and b) have indicated the same conclusion. Possible relationships between the peroxidase system and the DOPA-oxidase system are suggested, since the pattern of inactivation of both systems is similar in the case of leucocytes and erythrocytes. The DOPA reaction of these cells has usually been considered to be due to a phenol oxidase but van Duijn's results suggest that it is partly due to the action of these cells in trapping coloured autoxidation products from the incubating medium. One is bound to conclude that although the DOPA reaction can still be used in applied histochemistry interpretation of the results obtained is at present subject to grave uncertainties.

Histochemical Applications. Improvements in the technique of the DOPA reaction were reported by Redaeli (1953) and Rappaport (1955) described a semiquantitative application to freeze-dried skin. Kukita and Fitzpatrick (1955) used an entirely different method for demonstrating tyrosinase depending on autoradiography and radioactive tyrosine. Foster and Cook (1954) carried out a study of tyrosinase in mouse skin and Comstock and Wynne (1957) discussed the detection of the DOPA

factor in pigmented tissues. The phenol oxidase systems of human melanomas have been studied by Kertész (1954) and Fitzpatrick (1952) described the presence of an inhibited tyrosinase in normal skin, which was activated by irradiation. In junctional nævi he found a partially inhibited system and in melanomas, both pigmented and amelanotic, a fully active system.

The non-specific "DOPA-oxidase" of neutrophil and eosinophil leucocytes and myelocytes has not been the object of much attention. Vercauteren (1951) demonstrated the presence in horse eosinophil leucocytes of an enzyme system capable of oxidizing DOPA and he showed that this was inhibited by 10^{-2} M-KCN. Similarly the DOPA reaction of erythrocytes has produced little comment although many workers (e.g. Miescher, 1923; Moncorps, 1924; Bloch and Peck, 1930; Meirowsky, 1940) have noted it in passing. Erythrocytes and eosinophils from horse blood were used by van Duijn (1957b) in his inactivation studies. As mentioned above, he produced strong evidence of the identity of the DOPA factor with peroxidase or peroxidase-like activity in both cell types. Extensive tests were carried out by Lillie (1956) on the nature of phenol oxidative activities in skin. He found that the so-called DOPA (tyrosinase) system was localized at the presumed sites of cutaneous melanogenesis and that oxidation of *p*-aminodimethylaniline was situated in hair medulla and sheath cells. Activity against catechol, pyrogallol and hæmatoxylin was found in keratohyalin and trichohyalin granules. Although the DOPA-oxidase reaction was inhibited by 0·001 M-cyanide, sulphide or azide, the other activities withstood ten times these concentrations of inhibitor. Autoxidation was not completely excluded as responsible for some of the observed effects, especially those resulting in the staining of keratohyalin by polyphenol dyes like hæmatoxylin.

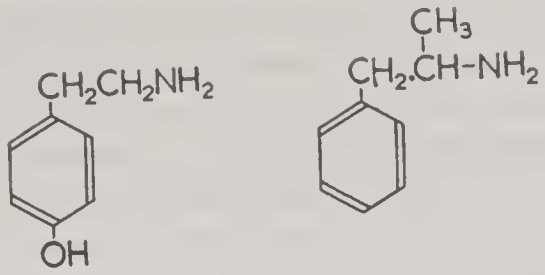
Metal-free Oxidases

The Biochemistry of Monoamine Oxidases

These group specific enzymes occur in a variety of animal tissues in small concentrations. They act on primary, secondary and tertiary mono-amines with the formation of either lower amines or aldehydes. On primary amines their action may be described as follows:

$$R.CH_2NH_2 + H_2O + O_2 \rightarrow R.CHO + NH_3 + H_2O_2$$

Two substrates upon which monoamine oxidase acts particularly rapidly are tyramine and adrenalin, and it is inhibited by similar monoamines in which there is a substituted methyl group in the α position, e.g. benzedrine.



Tyramine Benzedrine

Most animals possess a high amine oxidase activity in liver and kidney, rat kidney being an exception in this respect (Blaschko *et al.*, 1937 ; Pugh and Quastel, 1937). Langemann (1951) has reported high activity in the ox adrenal medulla and (1944) a moderate amount in human brain and heart muscle. According to Zeller (1951) an inverse relationship is always observed between amine oxidase and cholinesterase, especially in the brain.

The important functions carried out by amine oxidase in the animal body probably include the detoxication of poisonous amines produced in the intestine by bacterial decarboxylation of amino-acids, and a major part of the destruction of adrenalin, at adrenergic nerve endings and elsewhere. Recently several groups of workers (Blaschko and Philpot, 1953 ; Blaschko and Hellmann, 1953 ; Titus and Udenfriend, 1954 ; Udenfriend *et al.*, 1956 ; Sjoerdsma *et al.*, 1955) have shown that serotonin (5-HT) is deaminated to 4-hydroxyindole acetaldehyde by monoamine oxidase and this is subsequently excreted in the urine as 5-hydroxyindole acetic acid.

The exact pathways by which amines are oxidized in mammalian and plant tissues are improperly understood, however, and until

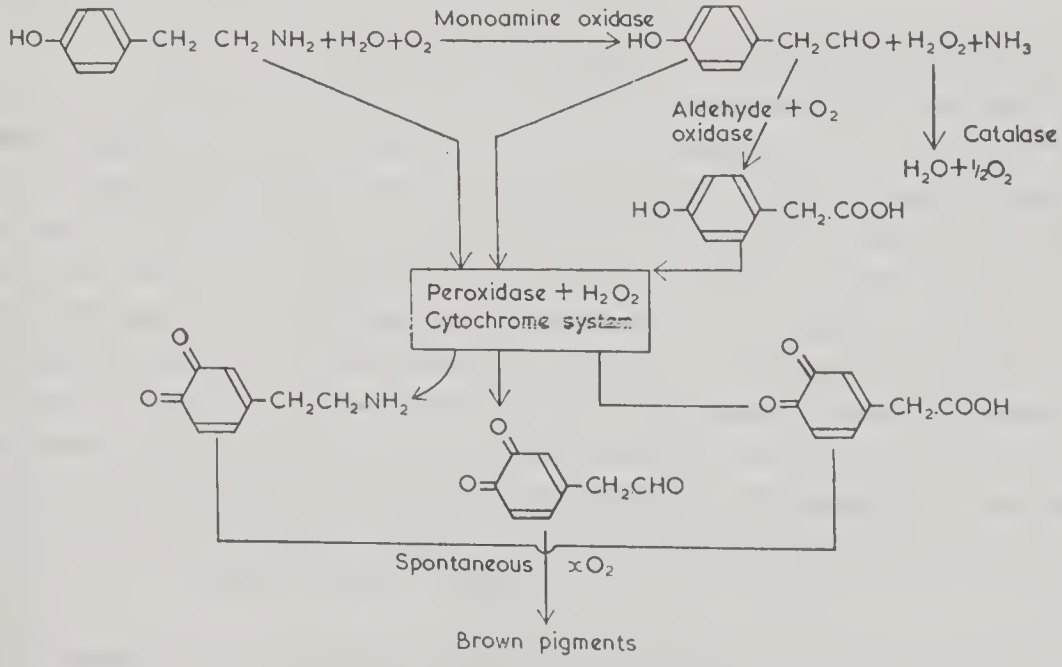


FIG. 171. Possible Oxidation Pathways for Tyramine. (After Creasey.)

recently there was little or no proof of the aldehydic nature of the presumed primary reaction product. Considering the factors which might interfere with the biochemical assay of monoamine oxidase activity Creasey (1956) suggested that a number of other enzymes might play an auxiliary role in the process. These included aldehyde oxidase, cytochrome oxidase, catalase and peroxidase. The whole scheme is shown in Fig. 171 on p. 527.

In the case of the washed mitochondrial suspensions which he used Creasey found no evidence that cytochrome oxidase played any part in the oxidation of tyramine and he could not demonstrate the presence of any aldehyde oxidase activity. Since this is a soluble enzyme it is probable that it was removed during the washing process. In the absence of a suitable substrate no peroxidase activity occurred but if a small quantity of ethanol was added a coupled oxidation took place, utilizing H_2O_2 produced in the primary reaction. In view of these findings Creasey considered that spontaneous aerial oxidation of *p*-hydroxyphenyl acetaldehyde could account for the extra O_2-uptake observed in manometric studies. It was found possible to prevent this in several ways. Creasey was unable to prepare *p*-hydroxyphenyl acetaldehyde but Clarke and Mann (1957) successfully isolated 3-indolyl acetaldehyde produced by the action of plant amine oxidase on tryptamine. Their work has shown that previous failure to identify this oxidation product was due to secondary oxidation catalysed by other enzymes. In the presence of added H_2O_2 they found that both tryptamine and 3-indolyl acetaldehyde were oxidized by peroxidase.

The observations recorded above are obviously of considerable histochemical importance. This should become clear in the following section.

Histochemical Methods for Monoamine Oxidase (DW. 233)

The first histochemical method for monoamine oxidase (MAO) was that of Oster and Schlossman (1942). This depended on the ability of fresh frozen sections of guinea-pig kidney to produce aldehyde when incubated with a 0·028M solution of tyramine in 0·06M-phosphate buffer (pH 7·2) for 24 hours at 37°. After incubation the sections were immersed in Schiff's solution until the maximum colour developed. In order to prevent the reaction of tissue aldehydes already present the sections were treated, before incubation, with a 2 per cent. sodium bisulphite solution (see Chapter XII, p. 341). Although this manœuvre successfully bound the original tissue aldehydes it was probable that more developed through oxidation of lipids during the long incubation period. Control sections, incubated in the absence of tyramine, were also used by the original authors.

Gomori (1950) severely criticized the specificity of localization given by this method ; he also objected to the stress laid by Oster and

Schlossman upon the distinction between the blue colour given by their reaction and the usual purple-red which develops when tissue aldehydes react with Schiff's solution.

In the first edition of this book I agreed with Gomori that the method could not localize the sites of enzyme activity. The reaction product was soluble in water and diffused freely in the incubating medium. It behaved then like any other aldehyde under similar conditions and became attached to polar groups such as NH_2, NH and SH in the tissue proteins ; since the pH of the medium is above 6, such attachment was probably mainly to NH_2 groups. The presence of a blue colour in the sections did not therefore indicate the localization of amine oxidase, but only some of the sites of attachment of the reaction product to protein.

In view of these observations I suggested that a simultaneous coupling method might be designed which would trap the aldehyde at the moment of its release to form an insoluble precipitate *in situ*. Attempts which I made to achieve this object by using 0·003M-tyramine, at pH 7·4, and 37°, in the presence of 0·005M-2-hydroxy-3-naphthoic acid hydrazide, were not entirely successful. Final treatment with *o*-dianisidine at pH 7·4, however, revealed a bluish-purple deposit in tissues containing amine oxidase which was largely absent from control tissues incubated in the absence of tyramine. Tissues were pretreated with hydroxylamine hydrochloride (Chapter XII, p. 344) to bind pre-existing aldehydes and sections thus treated gave a negative reaction when incubated in the absence of substrate.

Development of Naphthoic Hydrazide Methods

A considerably improved method, using the naphthoic acid hydrazide capture reagent, was developed by Koelle and Valk (1954). Instead of hydroxylamine they used 0·01M-hydrazine as their aldehyde-blocking reagent although it was observed that in many tissues no blocking reagent was necessary. In place of tyramine they employed tryptamine as substrate and found a considerable increase in the intensity and sharpness of staining. A third modification was the incorporation of 20 per cent. sodium sulphate into the medium, which was saturated with respect to naphthoic acid hydrazide. Throughout the incubation period oxygen was bubbled through the medium. Control sections were incubated in the absence of substrate and in the presence of the specific MAO inhibitor 1-*iso*nicotinyl-2-*iso*propylhydrazine (Marsilid, 0·001M). The latter completely abolished the histochemical reaction.

While the Koelle-Valk procedure is fairly satisfactory for the demonstration of MAO in the majority of mammalian tissues it is not possible

to use the method on central nervous tissues or on tissues containing much oxidizable lipid. In these a pseudoplasmal reaction with the hydrazide results in deeply stained control sections.

The Koelle-Valk procedure was employed by Eder (1957) in an extensive study of the distribution of MAO in guinea-pig tissues. Directions given in Appendix 18, p. 905, are derived mainly from the original paper of Koelle and Valk, with few modifications. Even in tissues with low MAO activity it is possible to obtain a positive reaction. Fig. 172, p. 521, shows the result obtained in a thin (8μ) section of rat kidney. This organ is known to possess a low MAO content. It is reasonable to employ sections thicker than 8μ in most cases since the reaction cannot be expected to give precise intracellular localization. The kinetics of the capture reaction are unknown and it is probable that first order conditions (see Chapter XIII, p. 373) are not attained. The solubility of naphthoic acid hydrazide is low but substitution of its more soluble hydrochloride by Glenner, Burtner and Brown (1957) produced relatively little improvement. It did, however, permit the omission of sodium sulphate from the incubating medium and the period of incubation was consequently lowered to one hour.

Methods Based on Pigment Formation

The original technique using the principle of pigment formation was that of Blaschko and Hellmann (1953) who incubated thick (25μ) fresh frozen sections with tryptamine hydrochloride at pH 7·4. A dark-brown pigment was formed after one hour's incubation. The authors noted that accelerated pigment formation occurred if H_2O_2 was added to the medium and that *sec*-octyl alcohol, which inhibits MAO, made no difference to the amount of pigment produced. A slight modification of Blaschko and Hellmann's method was used by Arioka and Tanimukai (1957) in a study of MAO activity in mouse brain. They used 5-hydroxytryptamine as substrate and heavy metals in trace amounts as additional catalysts. Their controls (heated sections ; substrate-free medium) showed no pigment formation.

In my opinion methods using this principle cannot be expected to give accurate localization of MAO and, in fact, pigment may be produced from aromatic amines by other enzyme systems in the absence of MAO. A glance at Fig. 171, p. 527, shows that the aldehyde intermediates from aromatic amines may be oxidized to pigments in a variety of ways. Since these intermediates are soluble compounds they can be expected to diffuse away from the site of production even if aerial oxidation, and not a second enzyme system, is the operative capture reaction. In the absence of catalysts the latter will probably not obey first order kinetics. If other enzyme systems are concerned in the conversion of intermediate reaction products to pigments then the locali-

zations indicated will be those of the secondary enzyme system. In the illustrations provided by Arioka and Tanimukai pigment deposition, described as in the capillary walls, appears rather to involve their contained red cells. The peroxidase activity of hæmoglobin is a factor which complicates a number of histochemical reactions for enzymes (cf. indoxyl esterase, Chapter XVI, p. 470). Accentuation of pigment formation after addition of H_2O_2, observed by a number of workers, is also to be regarded as indicating secondary oxidation by peroxidases.

In many cases the site of activity of the secondary enzyme system may be closely related to the site of MAO activity and in such cases the final localization may well be that of MAO itself. In view of the doubts which necessarily exist, however, I consider that methods for MAO based on pigment formation should not be used in applied histochemical studies.

Methods Based on Reduction of Tetrazolium Salts

The first use of this principle for the demonstration of MAO was reported by Francis (1953) who incubated 40μ sections of guinea-pig kidney with tyramine at pH 7·4 in the presence of 0·1 per cent. neotetrazolium chloride. After 2–3 hours at 37° a purple deposit of formazan occurred and this was regarded as being due to MAO activity since negative control sections were obtained if the substrate was omitted. The reaction was also observed to be inhibited by octyl alcohol and was presumably due to reduction of the tetrazolium salt by *p*-hydroxyphenyl acetaldehyde. In the hands of most workers the Francis method was unsatisfactory. By substituting tryptamine for tyramine and by using a more easily reducible tetrazolium salt Glenner *et al.* (1957) were able to obtain excellent results in suitable tissues after one hour's incubation. In their experiments total inhibition was produced by preincubation either with Marsilid (1-*iso*nicotinyl-2-*iso*propyl hydrazine) or with phenylhydrazine. Octyl alcohol produced no inhibition unless included in the medium, when partial inhibition occurred. Formazan pigment was produced under histochemical conditions from iodonitrotetrazolium (INT) and from nitro-blue tetrazolium (Nitro-BT) but not from neotetrazolium or blue tetrazolium. A glance at the table of redox potentials (Chapter XIX, p. 539) will indicate the reason for this observation and for the failure of the Francis method in the hands of most workers. Since the formazan from INT is macrocrystalline Nitro-BT is the reagent of choice in the histochemical performance of the MAO reaction.

The mechanism by which tetrazolium salts are reduced in the MAO reaction has been investigated by Weissbach *et al.* (1957). Their studies showed that *in vitro* INT was reduced only when an indole amine was oxidized by the MAO of rat liver mitochondria. Other substrates for

MAO did not give rise to products capable of reducing tetrazolium salts. Glenner *et al.* (1957) considered the possibility that electrons from the oxidized substrate might be transferred to the tetrazolium salt via the diaphorase system of the mitochondria. There is no evidence to support this hypothesis, however, and it seems more likely that the indole carbonyl primary reaction product is able to reduce the tetrazolium salt directly provided that the latter has a sufficiently high redox potential.

It is probable that most future studies on MAO will be carried out with the tetrazolium reactions, details of which are given in Appendix 18, p. 906, but if doubts of the specificity of the localization obtained arise it is reasonable to use the naphthoic hydrazide method as a control procedure.

Method for D-Amino-acid Oxidase (DW. 222)

This enzyme is classified as an oxidase since its natural hydrogen acceptor is molecular oxygen, which is reduced to hydrogen peroxide. It is able to use methylene blue as an acceptor, however. The enzyme acts only on members of the non-naturally occurring D-series of amino-acids but is able to attack most of the members of this group. Oxidative deamination takes place in two stages. In the first, which is catalysed by the enzyme, an imino-acid and H_2O_2 are the products. In the second, which is spontaneous, an α-keto-acid and NH_3 are produced.

It has been reported by Farber, Sternberg and Pearce (1958) that phenazine methosulphate is able to accept electrons in the D-amino-acid oxidase system and to pass them on to suitable tetrazolium salt such as Nitro-BT. In rat kidney the enzyme was observed to be concentrated in the second portion of the proximal convoluted tubule. The critical factor in the histochemical performance of this reaction appears to be the phenazine methosulphate. With some samples no reaction whatsoever can be obtained. Further studies on this enzyme system and its mechanism are necessary.

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CHAPTER XIX

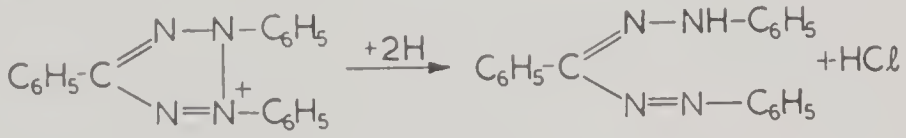
PRINCIPLES OF DEHYDROGENASE HISTOCHEMISTRY

There are a number of important differences between reactions for hydrolytic enzymes and those which demonstrate the various dehydrogenase systems. This chapter is therefore devoted to the principles on which the modern histochemistry of the dehydrogenases is based. These are of such importance in applied histochemistry that no further excuse is necessary. Nevertheless, much of what has already been stated in Chapter XIII is relevant here and reference will be made back to this chapter at appropriate points.

The chief difference between dehydrogenase methods and hydrolytic enzyme methods is that whereas with the latter the natural substrate is rarely employed, in the dehydrogenase methods its use is invariable. All the factors taking part in the reaction can be those which are concerned with the reaction *in vivo* except for the presence of a tetrazolium salt. Our primary concern therefore is with the nature of the tetrazolium reaction and with the characteristics of tetrazolium salts and their formazans. After this we have to consider the kinetics of the various dehydrogenase reactions and the participation of the various components of the respiratory chain.

The Development and Early Use of Tetrazolium Salts

The first tetrazolium salt, triphenyl tetrazolium chloride (TTC) was prepared by von Pechmann and Runge in 1894 as a pale yellow, water-soluble, crystalline powder. Kuhn and Jerchel (1941) found that a number of colourless tetrazolium salts were reduced to coloured compounds by plant tissues according to the equation given below.



Tetrazolium salt (Colourless)

Formazan (red)

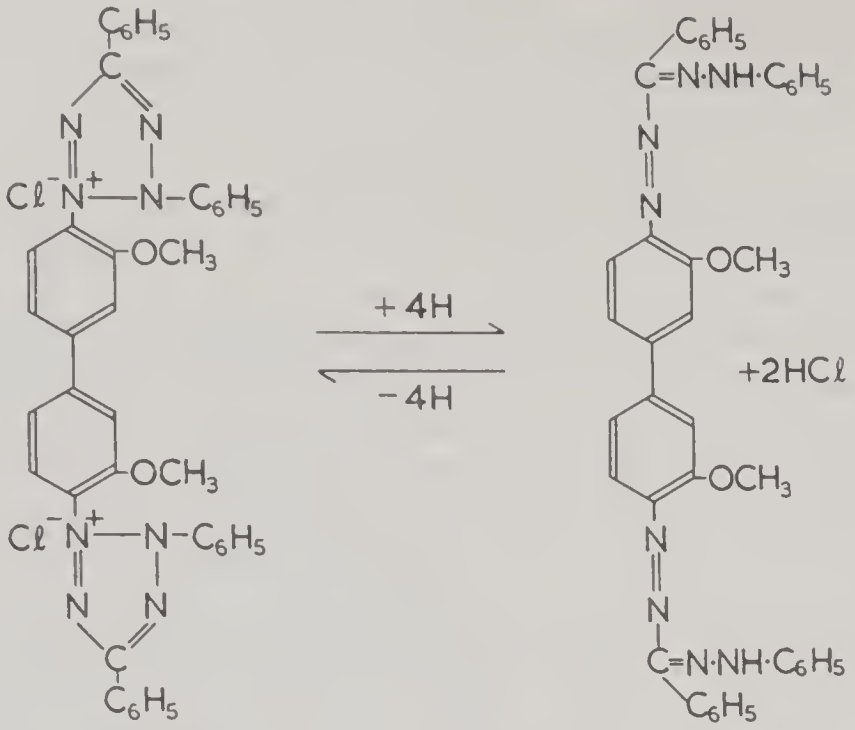
They noted that this effect was not due to the usual reducing substances present in the cells (glutathione, ascorbic acid, cysteine) because these substances would not reduce tetrazolium salts below pH 9·0. The characteristic reduction took place, in plant tissues, at pH 7·2. Mattson, Jenson and Dutcher (1947) showed that reduction of TTC was not due to reducing sugars, since these were ineffective

below pH 11·0, and Lakon (1942) used TTC as a test of the viability of seeds by observing the production of the red formazan in viable tissues. When tissues heated at 80° for short periods were found to be inactive, it was realized that some enzyme system in the tissues was responsible for the effects observed and Mattson *et al.* suggested that the reduction of TTC was due to dehydrogenase systems requiring Coenzymes I or II. According to Kun and Abood (1949), however, TTC revealed the presence of succinic dehydrogenase, which does not need a coenzyme, while Dufrenoy and Pratt (1948) suggested that the sites of reduction of tetrazolium salts were also the sites of reaction of phosphate ions. The effect of various inhibitors on the reduction of TTC by normal plant tissues was studied by Roberts (1951) who claimed that the formazan pattern of TTC was identical with that obtained with the Bennett sulphydryl reagent (Chapter V, p. 104).

It soon became clear, however, that tetrazolium salts were acting as electron acceptors in enzyme-catalysed oxidations in the same way as methylene blue. A histochemical method for succinic dehydrogenase depending on the conversion of the latter to leuco-methylene blue was indeed proposed by Semenoff (1935). Diffusibility of the unreduced dye made localization by this method impossible.

A number of authors used TTC and related compounds in the study of animal tissues (e.g. Straus, Cheronis and Straus, 1948 ; Antopol, Glaubach and Goldman, 1948 ; Black and Kleiner, 1949 ; Black, Opler and Speer, 1950 ; Seligman, Gofstein and Rutenburg, 1949). Histochemically the difficulty of obtaining a positive reaction with TTC, and the weak colour and diffusibility of its formazan in the tissues, were serious objections to its continued use. Efforts were therefore made to overcome these objections by the synthesis of new compounds and of these three gave results which were sufficiently improved to carry dehydrogenase histochemistry forward on the next stage of its development. These compounds were 2,2′-(*p*-diphenylene)-bis-(3,5-diphenyl) tetrazolium chloride, known as *neotetrazolium* or NT ; the methoxy derivative of NT, called *blue tetrazolium* or BT ; and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride or INT. The first was used by Antopol *et al.* (1948) and the second by Rutenburg, Gofstein and Seligman (1950). The monotetrazolium salt INT was produced by Atkinson *et al.* (1950), and this compound was found to be much more easily reduced than any other salt available at that time. The two ditetrazolium salts, however, were found to give better histochemical results and almost all the work in the field of dehydrogenase histochemistry between 1950 and 1958 was carried out with either NT or BT.

The reduction of BT takes place in the same way as the reduction of TTC except that a blue diformazan is the end product instead of a



Ditetrazolium salt (colourless) Diformazan (blue)

red monoformazan. The above equation indicates complete reduction ; partial reduction can occur at one end of the molecule only, giving a monoformazan which is reddish purple and more soluble than the product of complete reduction (see below). Two other tetrazolium salts which have been used in dehydrogenase histochemistry since 1957 are 2,2′-di-*p*-nitro-phenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro-BT) (Tsou *et al.*, 1956), and 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) (Pearse, 1957). The development of these, and a description of their main characteristics, is considered in a later section of this chapter.

Physical and Chemical Characteristics of the Tetrazolium Salts

Redox Potentials. The function of the tetrazolium salt in dehydrogenase histochemistry is to act as acceptor of electrons from the oxidized substrate. These electrons, if not intercepted, would normally pass by way of a succession of carriers to molecular oxygen. One of the main characteristics of the tetrazolium salt, which determines whether or not it will be suitable for histochemical purposes, is the ease with which it is reduced. This characteristic is the redox potential (E°) which is synonymous with the oxidizing capacity and is expressed in volts with reference to the normal hydrogen electrode. It can be measured by means of a potentiometer or recorded polarographically.

The earliest polarographic studies of the redox potential of TTC were those of Jerchel and Möhle (1944). Further work on mono- and ditetrazoles has been carried out by Ried and Wilk (1953), Jambor

(1954), Campbell and Kane (1956) and by Kivalo and Mustakallio (1956). These last authors included BT and NT in their survey.

Polarograms of tetrazoles and tetrazolium salts exhibit a number of reduction waves corresponding to the production of formazan, hydrazidine and amidrazone in succcssive stages. The first two waves to occur, which are often called the α and β waves, were considered by Campbell and Kane to be associated with the opening of the tetrazolium ring. The α wave was believed to be characteristic of reduction under special conditions where the product is absorbed on to the mercury surface below the dropping mercury electrode. Since this condition is not present in the biological systems with which we are concerned the α wave, when present, can certainly be ignored. Although the β wave involves reduction beyond the formazan stage Campbell and Kane consider that the half wave potential of this wave can be regarded as a measure of the ease with which a tetrazolium salt is reduced to its formazan. The half wave potential of the β wave is thus the parameter with which we are here concerned.

TABLE 44

Half Wave Potentials of Tetrazolium Salts and Tellurite

Oxidant	E $\frac{1}{2}$, 22°, pH 7·2, Volts.
Tellurite	—0·95
TTC	—0·49
NT	—0·17
BT	—0·16
MTT	—0·11
INT	—0·09
Nitro-BT	—0·05

The β wave half wave potentials shown in Table 44 were obtained with a Tinsley Recording Polarograph and dropping mercury electrode. The tetrazolium salts were dissolved in 0·1M-phosphate buffer at a concentration of 10^{-4}M. They were the purest available samples but the figures should nevertheless be regarded as accurate only for comparative purposes.

Tellurite was used by Wachstein (1955) as an indicator of so-called endogenous dehydrogenase activity in sections of human kidney. It has also been used as an indicator of succinic dehydrogenase activity in bacteria (Mudd, Takeya and Henderson, 1956) and in mammalian tissues (Barrnett and Palade, 1957). With the latter the incubation period is very long indeed and it is perhaps surprising that any result at all is obtained.

When we come to the tetrazolium salts we see (Fig. 173) that the half wave potential for TTC is —0·49v.

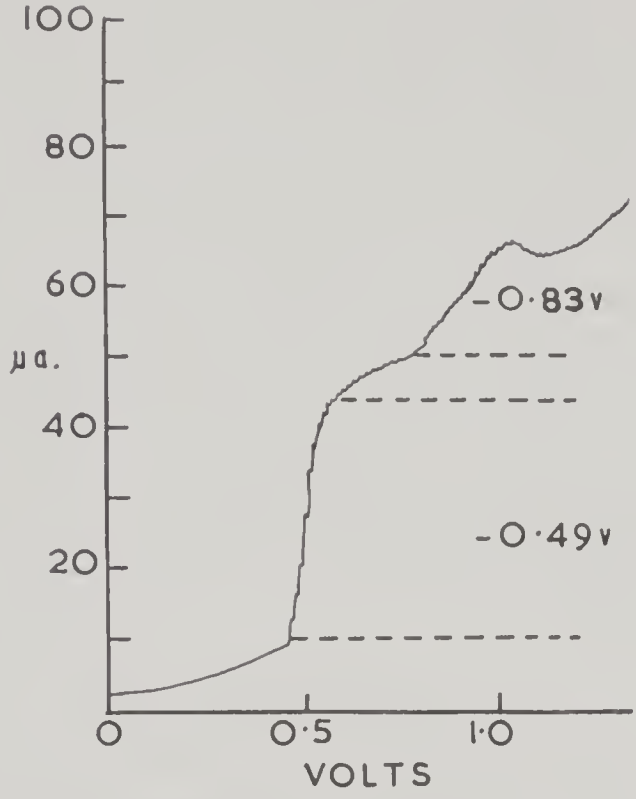


Fig. 173. Polarogram of Reduction of TTC.

This agrees closely with the result calculated from the traces for pH 7·6 given in their paper by Kivalo and Mustakallio but differs from the figures for pH 6·7 given by Campbell and Kane, which are approximately 0·1 volts higher. This difference is probably entirely due to the difference in pH since tetrazolium salts are more easily reduced at lower pH levels. The figure recorded for TTC is well below those for NT and BT, which are practically identical. In histochemical practice, however, NT is always observed to be more easily reduced than BT and on this account it has been preferred by many workers. In this instance, therefore, the half wave potential fails to reflect accurately the differences observed in biological systems. Nevertheless it seems to be significant that the three most easily reduced tetrazolium salts in common use (INT, MTT, Nitro-BT) have half wave potentials which are significantly higher than those of the other salts.

Figures 174 and 175 are reproductions of polarographic traces obtained with MTT and Nitro-BT respectively.

The comparative steepness of the curve given by Nitro-BT is especially notable and, in practice, this salt is found to be considerably more easily reduced than MTT. The important question of the relationship between the half wave potential of the β wave for a given tetrazolium salt, and the E° of the reaction giving rise to the electrons accepted by

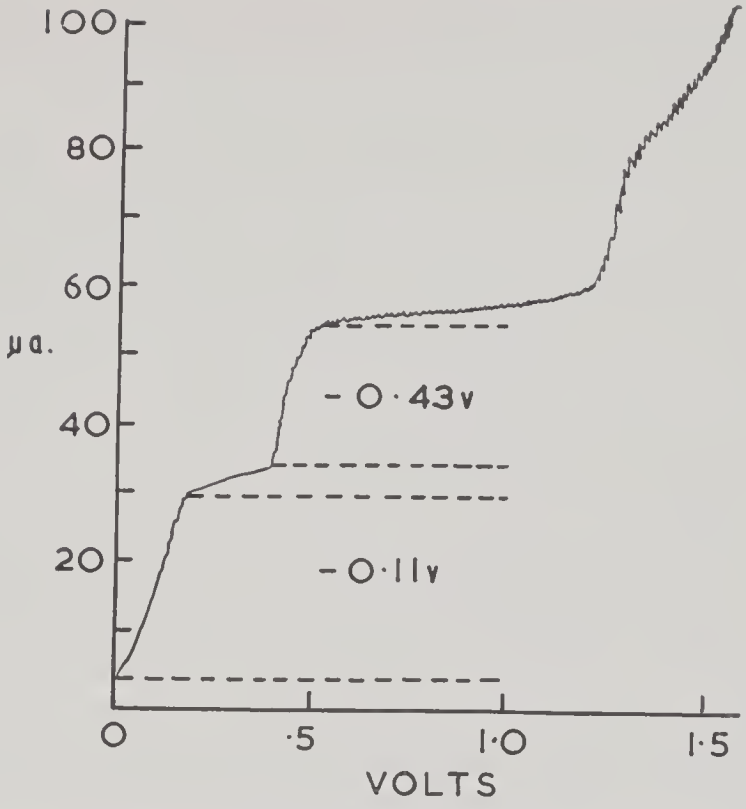


Fig. 174. Polarogram of Reduction of MTT.

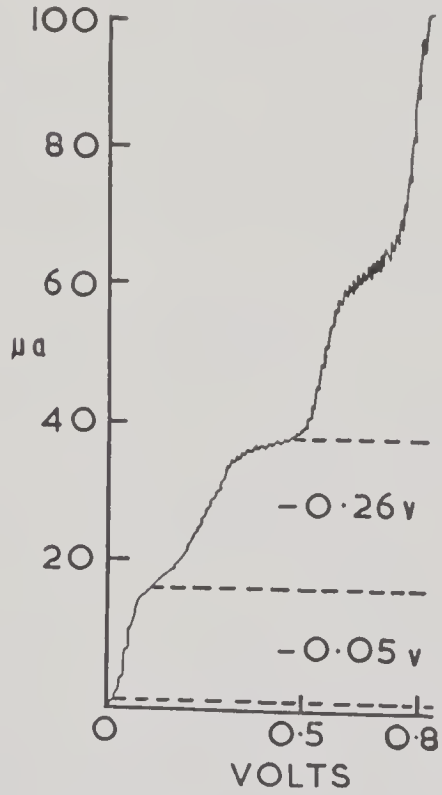


Fig. 175. Polarogram of Reduction of Nitro-BT.

this salt, is discussed in a later section of this chapter. It is already clear, however, that the half wave potential of a tetrazolium salt can be too high for it to be employed safely as an electron acceptor in dehydrogenase histochemistry. The precise limits of safety have not

been delineated but salts which are as easily reduced as Nitro-BT must be regarded as coming close to the limit even if they do not actually exceed it in some cases.

Solubility, Molecular Size and Charge. Most of the currently employed tetrazolium salts are sufficiently soluble to be used at their optimal concentration if required. Few studies have been made with the object of determining the latter but Glick and Nayyar (1956) found that for rat liver sections and INT the optimal concentration of the tetrazolium salt was between 4 and 6 mM., or 2 to 3 mg./ml. in the incubating medium. This figure is well above that usually employed in dehydrogenase histochemistry.

The molecular size of all tetrazolium salts so far employed is sufficiently small for the diffusion constant to be high enough theoretically to allow rapid penetration into the tissues (see Chapter XIII, p. 372). In practice, however, the *intact* membrane of the mitochondrion constitutes a barrier to the penetration of the tetrazolium salt so that the larger ditetrazolium salts may penetrate slowly or not at all. A low concentration of tetrazolium salt at the site of electron transfer may thus be the rate limiting factor even if the concentration in the medium is perfectly adequate.

In fully protected media (Chapter XIII, p. 367) the penetration of Nitro-BT into the mitochondrion is reduced to a low level and the amount of formazan is correspondingly small. It is necessary, in fact, for the mitochondrial membrane to be damaged before an adequate result can be obtained with Nitro-BT in the case of enzyme systems localized within this organelle.

Another factor which may seriously interfere with penetration of the tetrazolium salts is the possession of strong polar groups and/or substantivity for protein. Wattenberg (1954) found that when sections were incubated in the presence of low concentrations of a tetrazolium salt the latter was firmly bound to protein. This was shown by the fact that, after thorough washing, the application of a reducing agent produced a diffuse deposit of formazan throughout the section. Substantivity of the tetrazolium salt is thus an undesirable quality from the histochemical point of view. Lipid solubility, on the other hand, may be of value if it accelerates the rate of penetration through lipid or lipoprotein barriers. Measurements of the lipid solubility of tetrazolium salts do not appear to have been made ; MTT is certainly lipid soluble but Nitro-BT is not. The disadvantage of the latter with regard to penetration is certainly offset by the fact that progressive formazan production in lipid droplets does not occur. This process, when it occurs, is due to reduction of adsorbed tetrazolium by the autoxidation products of unsaturated fats.

Light Sensitivity. Many tetrazolium salts are sensitive to light and

undergo colour changes when left in the light for short periods. In the case of TTC two compounds are produced, one being the formazan and the other, 2,3-(2,2′diphenylene)-5-phenyl tetrazolium chloride, an oxidation product. It is fortunate that the tetrazolium salts most used in histochemistry are fairly stable to light and the thiazolyl tetrazolium salts (MTT) are exceptionally stable.

Inhibition of Enzyme Activity. Little appears to be known about the effect of different concentrations of tetrazolium salts on the kinetics of the dehydrogenase reactions. It is probable, however, that inhibition of enzyme activity is a factor which must be taken into account. It has been shown by Lettré and Albrecht (1943), Jerchel and Fisher (1949), and by Rutenburg *et al.* (1950), that tetrazolium salts are highly toxic to fibroblasts in tissue culture and to intact animals. Pearse, Scarpelli and Hess (1959) compared the values for oxidation of succinate, β-hydroxybutyrate, and DPNH and TPNH by isolated submitochondrial particles, obtained by estimation of formazan and by measurement of the oxygen uptake. The formazan values were about one-tenth of the values obtained from oxygen uptake. The most likely explanation of these findings is an inhibition of enzyme activity.

Physical and Chemical Characteristics of the Formazans

Colour, Size and Shape of the Deposit. Formazans are intensely coloured compounds giving shades which vary from red to deep purplish black. They also show brilliant reflex colours. This property was used by Fleischhauer (1958) who examined sections containing NT formazan by polarized light. The golden reflex of the crystals made

TABLE 45

Absorption Spectra of Formazans

Formazan from	Absorption Maxima			
	λmax	εmax	λmax	εmax
TTC	290	10,000	405	30,900
INT	—	—	500	11,000
NT	—	—	587	14,400
BT	—	—	606	10,400
Nitro-BT	355	37,900	510	55,000
MTT	560	51,000	—	—
MTT-Co*	560	20,000	660	20,000
5-MTT	550	31,000	—	—
5-MTT-Co*	550	44,000	675	26,000
4-MTT	430	13,000	447	41,000
4-MTT-Co*	436	39,000	—	—

* Cobalt-chelated ; see subsection on chelation below.

them easily distinguishable under the microscope from other (non-crystalline) reaction products deposited in the tissues. Table 45 gives details of the absorption spectra of formazans from tetrazolium salts which have been or are being used in histochemistry. Some of these have been derived from the literature and the remainder are measurements carried out in my own laboratory.

Absorption with two peaks, in the red and violet bands, gives the formazans their typical colour and strong absorption in the red band is characteristic of all formazans suitable for histochemical purposes. There is also strong absorption in the ultra violet at about 250 to 350 mμ but little use is made of this property. These features are shown in Fig. 176, below, which is the absorption spectrum of TTC. The high

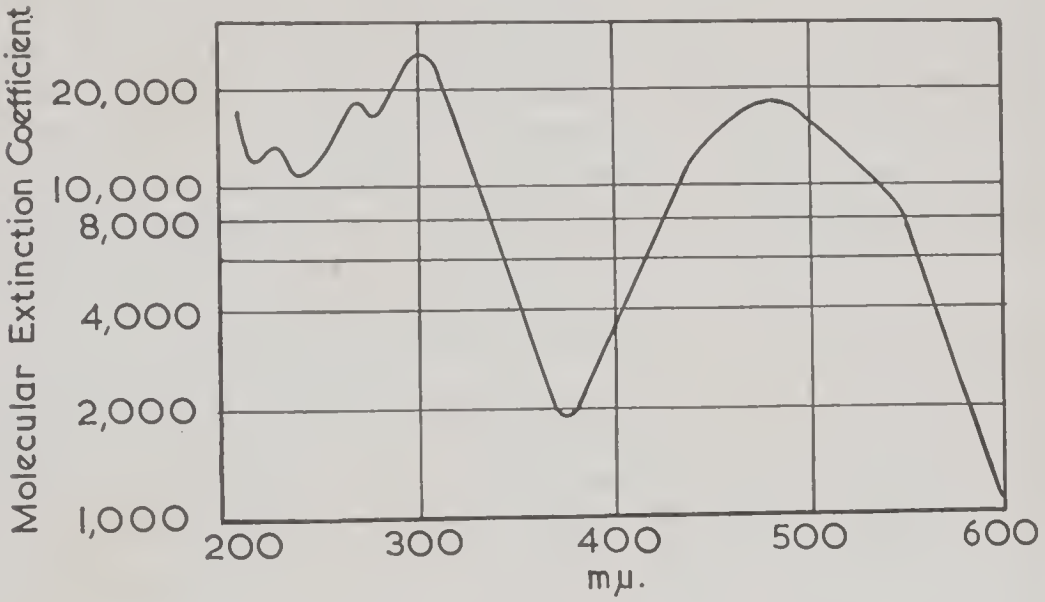
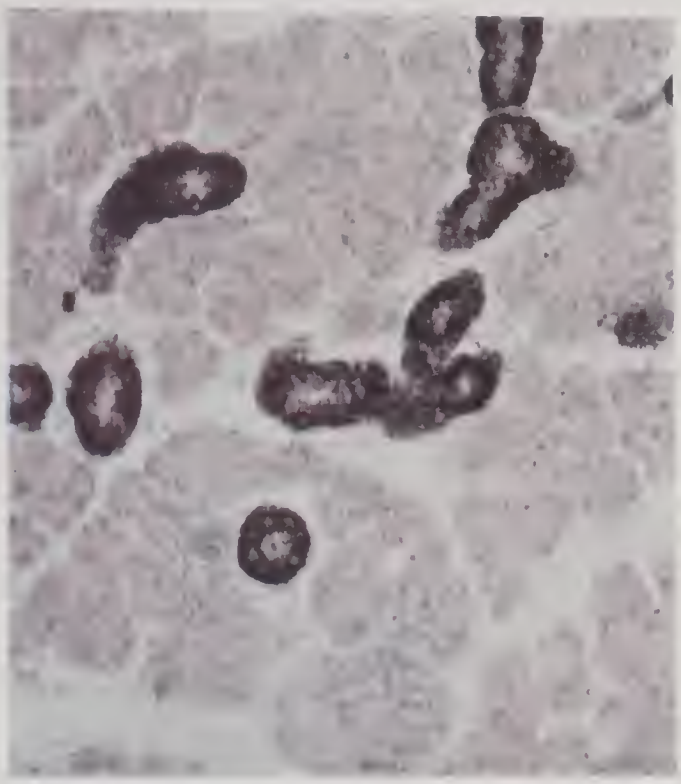


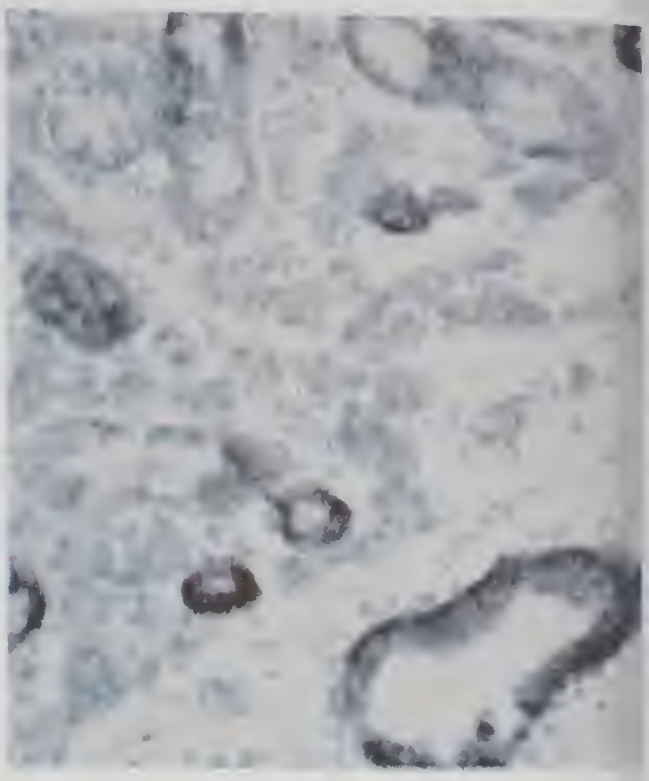
Fig. 176.

molecular extinctions of the diformazans are especially notable, particularly the diformazan from Nitro-BT, but the lower extinctions of the cobalt-chelated formazans from the 4,5-dimethyl and 5-dimethylthiazolyl tetrazolium salts are compensated for by the higher wavelengths at which maximal absorption occurs. When viewed by eye these cobalt-formazans appear black although they are actually deep purple in solution in organic solvents.

Half Reduction of Ditetrazoles. When it was originally noted that the ditetrazolium salts NT and BT gave rise to two different reduction products under histochemical conditions it was postulated that the blue product was the diformazan and the red product, occurring in areas of lower enzyme activity, was a monoformazan produced by reduction at one end of the molecule only. Later, however, Burtner, Bahn and Longley (1956) observed that in the case of NT the red derivative was probably the formazan from a contaminating monotetrazole. Monotetrazolium salts like TTC and INT are popularly

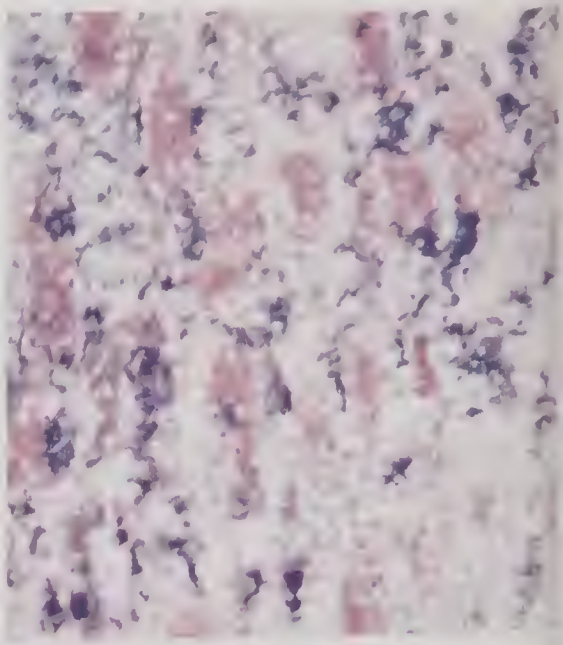


VIIA. Rat salivary gland. Shows high activity in duct lining cells but poor intracellular localization. Succinate dehydrogenase (Blue tetrazolium). × 200.



VIIB. Rat salivary gland. Great improvement in intracellular localization produced by using a salt giving rise to a substantive formazan. Succinate dehydrogenase (Nitro-blue tetrazolium). × 200.

VIIC. Rat kidney. Distal convoluted tubules. Similarly improved localization provided by an efficient capture reaction. Succinate dehydrogenase (Cobalt-tetrazolium, methyl green). × 700.



VIID. Rat kidney. Twenty-two days' potassium depletion. Shows TPN diaphorase in the thin loops of Henle (blue), in the ascending loops (pink), and in the collecting tubules (purple). Blue tetrazolium. × 130.

[*To face p.* 545.

supposed to have only one product. With several samples of INT I have repeatedly observed, however, that two formazans, a brick-red and a brownish-purple one, are deposited in tissue sections. This may, of course, be due to contaminating monotetrazolium salts though the distribution of the two formazans suggests otherwise. No information is available concerning the different coloured products from BT (see Plate VIIᴅ) but whatever their source this complication in the use of ditetrazolium salts constitutes a severe handicap to accurate interpretation of histochemical results.

In the case of Nitro-BT the position is still far from clear. All samples of this reagent at present available give rise to two reaction products, the purple diformazan and a red compound. The latter is invariably present, though in much smaller amounts than the diformazan. It is not known whether it is an intermediate reduction product.

Crystal Size. The monoformazans as a class are easily crystallized and they are usually deposited in the tissues in crystalline form. Many of the diformazans (e.g. those from NT and BT) are also deposited in tissue sections as crystals. The size of these varies from long needles $5 \times 1{\cdot}5\mu$ (INT) to short rods averaging $1{\cdot}5 \times 1\mu$ (BT). The crystal of NT diformazan is a longer rod measuring $2{\cdot}5 \times 1{\cdot}5\mu$. Crystalline products are not acceptable in dehydrogenase histochemistry since they make accurate localization impossible. The newer methods, which usually give rise to non-crystalline formazans, afford very much finer definition (see Plates VIIʙ and VIIc).

The metal formazans from thiazolyl tetrazolium salts can still form large crystals and, if the mitochondria in which they have been deposited are for any reason allowed to rupture (see Chapter XIII, p. 367 ; Protection of Mitochondria), diffusion of the formazan through the mounting medium allows aggregation and crystallization to take place. In the case of Nitro-BT the position is complicated. Its diformazan cannot be crystallized from solutions and initially crystals are not formed in the tissues. If, however, the sections are dehydrated in alcohols, cleared in xylene and mounted in a synthetic medium, aggregates and crystals of the diformazan or of a diformazan-protein complex are commonly observed. These, unfortunately, bear a close resemblance in size and shape to mitochondria but can be produced in model systems in which mitochondria are not present. For this reason I prefer not to dehydrate sections containing Nitro-BT formazan but to mount them in a watery medium.

Other Characteristics of the Formazans

Light Sensitivity. Many formazans on exposure to visible light are changed into red and yellow forms. The chemistry of the changes involved has been described by Hausser, Jerchel and Kuhn (1949). In

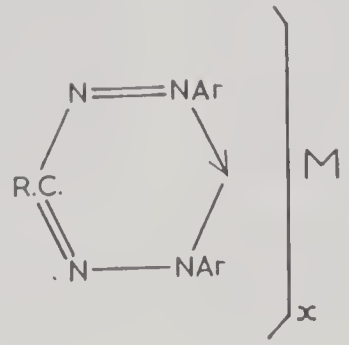
some cases the reaction is reversible when irradiation ceases but in others it is not. Light sensitivity is obviously an undesirable property from the histochemical point of view and it is thus fortunate that the majority of tetrazolium salts now employed give rise to stable formazans or diformazans.

Lipid Solubility. Formazans with aliphatic substituents (hydrogen, methyl, carboxyl) attached to the 3-carbon atom are very soluble in organic solvents and those with aryl substituents are somewhat less soluble. Triaryl mono-formazans are especially soluble in chloroform and acetone and also in triglycerides and phosphatides. This property is an embarrassing one from the histochemical point of view. The diformazans are much less lipid-soluble and the formazan from Nitro-BT appears to be completely insoluble in those lipids normally found in the tissues.

Substantivity for Protein. This property has been considered (Chapter XIII, p. 379) to be a desirable one in the case of the reaction product of a histochemical enzyme method provided that substantivity for a particular tissue component is avoided. The remarkable improvement in localization of dehydrogenase systems afforded by Nitro-BT is largely due to its general substantivity for protein which effectively prevents crystallization from occurring during incubation. It is interesting to note that in the case of the formazan from a closely related tetrazolium salt, 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′ (*p*-biphenylene) ditetrazolium chloride (Nitro-NT), substantivity for protein is lacking, as it is with all monoformazans so far tested. The property seems to be connected with the nature of the diformazan molecule which must be long and flat, without interfering substituent groups, so that it lies along the extended protein molecule to be attached to it by hydrogen bonding.

Metal Chelation of Formazans

Formazans are capable of forming metallic complex "salts", particularly with copper, nickel, cobalt and silver. These complexes were studied particularly by Hunter and Roberts (1941a and b) who prepared a series of metallic derivatives of formazans of the type shown below.

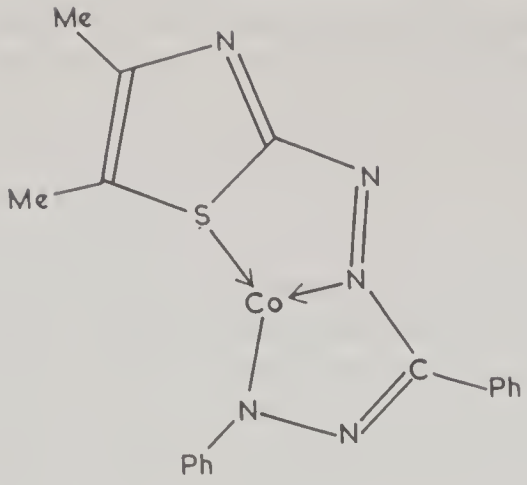


In these the imino-hydrogen atom of the formazan is replaced by one equivalent of the metal. Chelation with copper, nickel and cobalt took place only in hot acetone solutions, under reflux, and the products were stable, black pigments, insoluble in water, ethanol or light petroleum. Despite their high M.W., however, they were remarkably soluble in other organic solvents.

A search for formazans which would chelate under the milder conditions experienced in histochemical reactions (Pearse, 1957) resulted in the observation that chelation readily occurred with the formazans from N-thiazolyl-substituted tetrazolium salts (Beyer and Pyl, 1954). When thiazolyl tetrazolium salts are used as electron acceptors in dehydrogenase reactions in the absence of a chelator (Co^{2+}, Ni^{2+}, Cu^{2+}) the formazan deposits rapidly aggregate and crystallize (Fig.177 p. 560). In the presence of suitable metallic ions, on the other hand, the deposits are non-crystalline (Fig. 178 and Plate VIIc, p. 545). Thus a capture reaction has been established which prevents crystallization and which presumably also prevents diffusion of the free formazan. This observation forms the basis of the monotetrazolium-metal chelation methods for a variety of dehydrogenases and diaphorases. These are described in Chapter XX.

In my original paper on the subject (1957) a phenomenon called " escape " was described in which, after shorter or longer periods in storage, the dot-like formazan deposits began to crystallize out. The word " escape " suggested, though this was not intended, that the metal complex could break down to liberate the free formazan. There was never any direct evidence of this and further research has shown that breakdown of the complex is not responsible. Crystallization occurs if the metal-formazan is able to diffuse in the mounting medium (due to its solubility in organic solvents this has to be an aqueous medium) and this it can do if the mitochondria which contain it are damaged so that their membranes become permeable. " Escape " is not now observed in the case of cobalt-formazan deposits in intact (fully protected) normal mitochondria from all species so far examined.

The mechanism by which chelation of thiazol formazans takes place cannot be explained on the basis of the formula used by Hunter and Roberts. It is postulated, therefore, that cobalt is chelated by MTT and other thiazolyl formazans in such a way that a compound containing two additional 5-membered rings is produced. The metal is seen to be held by three co-ordinate bonds in the positions shown and the additional electron donor, in this case sulphur, is undoubtedly responsible for the ease with which the metal complex is formed under histochemical conditions. It should still be possible for the cobalt atom to accept an additional pair of electrons from another donor but whereas in Hunter and Roberts' formula x normally equals 2, with the



thiazolyl formazans the metal : formazan ratio should be 1 : 1. This might be an important point in connection with the development of quantitative techniques.

It is obviously necessary to determine the rate at which chelation takes place. Initial *in vitro* observations on the chelation of MTT-formazan, dissolved in organic solvents, by cobalt ions suggested that it was a relatively slow process (half time in the order of 500 millisecs). Using the data provided *in extenso* in Chapter XIII, it is evident that a rate such as this could not possibly provide an efficient capture reaction. It was therefore postulated that in aqueous media and under biological conditions a different rate obtains. Preliminary evidence from polarographic studies suggests that this is in fact the case and that cobalt chelation of the nascent formazan is much faster than with the formazan dissolved in organic solvents. It may well be found to be sufficiently rapid to account for the observed improvement in localization.

Objections to the Chelation Principle. So far only one important disadvantage attaches to the monotetrazolium-cobalt chelation methods. This is due to the fact that they cannot be employed when, for any reason, the presence of cyanide in the incubating medium is desirable. Cyanide may be required either in order to block the electron pathway to oxygen or, more importantly, as a trapping agent for carbonyl products resulting from the oxidation of various substrates. As will be seen in Chapter XX, dehydrogenase techniques in which cyanide is present in the medium must at present be carried out with a non-chelating technique, preferably using Nitro-BT.

The reason why Co^{2+} ions cannot be included in any medium containing CN^- and an electron acceptor (tetrazolium salt) is that an unstable cobalt cyanide complex is formed $[Co(CN)_6]^{4-}$, as described by Keller and Parry (1956), and this rapidly reduces the tetrazolium salt to its formazan.

Extensions of the Chelation Principle. Alternative possibilities for metal chelation of formazans under histochemical conditions were

suggested by the use of copper complexes of formazans with *ortho*-hydroxyl and carboxyl groups on the N-phenyl rings as dyes (Wizinger and Biro, 1949 ; Wizinger and Herzog, 1953). I explored this possibility but found that tetrazolium salts containing these groups possessed redox potentials which were too low to allow them to function as electron acceptors for dehydrogenase systems. The production of copper complexes of *o*-carboxyformazyl-2-quinoline has been described by Seyhan (1952, 1953). Tetrazolium salts from such formazans have not been used in biological systems.

Physicochemical Characteristics of Dehydrogenase Reactions

Kinetics. The dehydrogenase systems which we can demonstrate histochemically in a satisfactory manner are those in which the equilibrium point of the reaction lies well over to the right, that is towards oxidation of the substrate, or in which the equilibrium can easily be shifted to this side. This fact can be illustrated with reference to alcohol dehydrogenase.

$$\text{Alcohol} \underset{\text{DPNH}}{\overset{\text{DPN}^+}{\rightleftharpoons}} \text{Acetaldehyde}$$

In the case of yeast alcohol dehydrogenase the Michaelis constant (Km) for alcohol is $2{\cdot}4 \times 10^{-2}$ whereas for acetaldehyde it is $1{\cdot}1 \times 10^{-4}$ (Racker, 1955). The equilibrium of this system thus lies very far to the left, that is to say the natural tendency of the enzyme is to reduce acetaldehyde to ethanol.

The Michaelis Constant. According to Michaelis and Menten (1913) substrate concentration was to be regarded as an important factor controlling the velocity of an enzyme reaction. The velocity (v) is related to the substrate concentration (s) by the Michaelis-Menten equation

$$V = \frac{v}{I + \frac{Km}{s}}$$

where V is the maximum velocity of the reaction when the enzyme is saturated with substrate. The term Km is the Michaelis constant. If v is plotted against s, as in Fig. 179, below, the curve is a section of a rectangular hyperbola.

If we substitute $v = V/2$ then $s = Km$ and Km is thus the halfway point of the experimental curve or, in other words, the concentration of substrate required to give half the maximum velocity. It has been pointed out by Dixon and Webb (1958), whose book should be consulted

for the derivation of the Michaelis-Menten equation, that confusion arises through the original use of the term Michaelis constant to mean the dissociation constant of the enzyme-substrate complex. For this

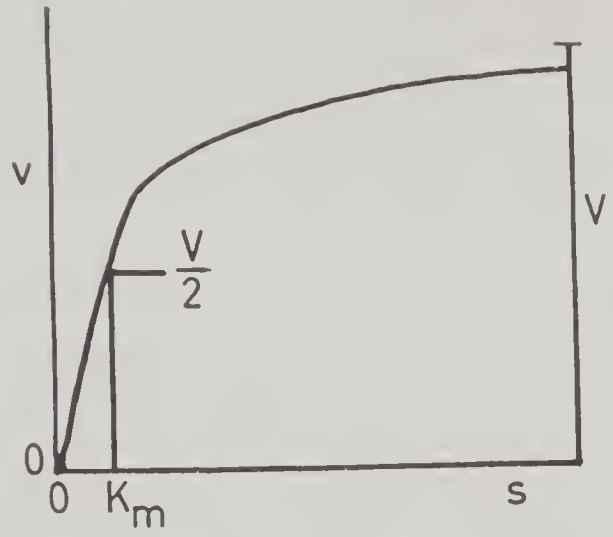


Fig. 179. Effect of Substrate Concentration.

they now propose the use of the term " substrate constant ", denoted by K_s, and the restriction of the term " Michaelis constant " to the definition given above. The relationship between Km and Ks can be made more clear by some additional information.

Enzyme catalysed reactions can be expressed in the following manner :

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

and after this essential combination of enzyme (E) with substrate (S),

$$ES\ (+ \text{ any second reactant}) \xrightarrow{k_3} E + \text{products.}$$

$$Km = \frac{k_2 + k_3}{k_1}$$

The dissociation constant (K_s) for the enzyme-substrate complex (ES) is equal to the Michaelis constant (K_m) only when k_3 is very small compared to k_2 ; then $K_m = k_2/k_1 = K_s$. In most cases the response of an enzyme reaction to changes in substrate concentration is related to the ratio of the latter to the Michaelis constant. Dixon and Webb use the term " relative substrate concentration " (σ) to express this and

$$\sigma = \frac{s}{Km}$$

They also define the term " relative velocity " as

$$\phi = \frac{v}{V}$$

and write the Michaelis equation as

$$\phi = \frac{\sigma}{1 + \sigma}$$

It therefore follows that when the substrate concentration is high $\phi = 1$ and when it is low $\phi = \sigma$, as should be evident from the curve shown in Fig. 180, below.

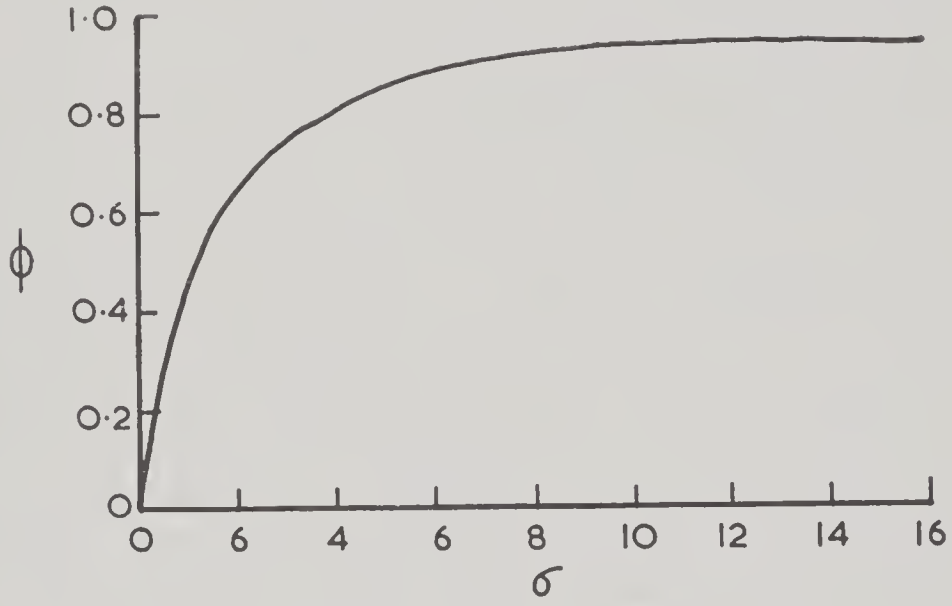


Fig. 180. Normalized Substrate Concentration Curve.

Other forms in which the Michaelis equation can be plotted are given by Dixon and Webb.

In applied dehydrogenase histochemistry the velocity of the forward reaction, and thus the Michaelis constant for the substrate of this reaction, is of paramount importance. The higher the Km value the lower the affinity of enzyme for substrate and the higher the concentration of substrate necessary to achieve a given forward velocity. In the hydrolytic enzyme field a low velocity may be quite acceptable and may indeed result in more accurate localization in cases where the capture reaction is relatively inefficient. With the dehydrogenases, however, we require the maximum possible velocity. This is partly due to the fact that prolonged incubation times damage the mitochondria and partly to the fact that when oxidation of the substrate falls to a low level alternative electron acceptors present in the tissues may absorb the entire supply of electrons, leaving the tetrazolium salt unreduced.

If we know the Michaelis constant for both sides of a dehydrogenase reaction the probability of the reaction proceeding in the required direction can be estimated. Following this the incubating medium can be designed to include the substrate at a sufficiently high concentration, provided that its solubility allows this.

Referring once again to the example of alcohol dehydrogenase we note that biochemists, who are more interested in the oxidation of alcohol than in the reduction of acetaldehyde, introduce aldehyde-trapping reagents into their media. These combine rapidly with acetaldehyde and remove it from the system which is thus prevented from coming to an equilibrium. The demand for a continuous supply of acetaldehyde then ensures that the reaction runs to the right.

Trapping Reagents. In a substantial number of important dehydrogenase reactions the product is either an aldehyde or a ketone. The equilibrium point of many of these reactions lies to the left rather than to the right and it is therefore necessary, in histochemical practice, to bring the equilibrium over to this side by increasing the substrate concentration to a high level and by removing the product with a carbonyl-trapping agent. There are a number of possible reagents for this purpose (see Chapter XII, p. 341) and biochemists have used, *inter alia*, bisulphite, cyanide, dimedone, phenylhydrazine and semicarbazide. The inclusion in histochemical media of hydrazine, hydroxylamine, phenylhydrazine or semicarbazide has no effect on the rate of reduction of the tetrazolium salt (Hess, Scarpelli and Pearse, 1958). These compounds in fact tend to inhibit dehydrogenases (Kaplan and Ciotti, 1953) and at concentrations higher than 0·01M they are able spontaneously to reduce tetrazolium salts even at a neutral pH. Cyanide, on the other hand, has been found to act as an effective trapping agent for carbonyl groups released under histochemical conditions. Two points must be mentioned in connection with its use, however. First, as noted above, cobalt chelation techniques cannot be used in the presence of cyanide though it may be possible to use other metals if these are satisfactory chelators. Secondly, cyanide has an additional important effect in blocking the electron transport system of the cell. This is more fully considered later in this chapter.

pH Optima. The effect of pH changes on the equilibria of reactions requiring pyridine nucleotide coenzymes is often profound, as in the case of alcohol dehydrogenases and small changes may alter the Km value significantly. In dehydrogenase histochemistry we cannot always work at the pH optimum of the enzyme system we are studying, particularly if this is in the alkaline range. This is especially true in the case of the pyridine nucleotide-linked dehydrogenases where the so-called " nothing dehydrogenase " activity rises sharply above pH 7·0. This is described in the next section of this chapter. If, therefore, a

DPN or TPN-dependent dehydrogenase has a narrow peak of activity on the alkaline side of neutrality, as has aldehyde dehydrogenase, we are unlikely to be able to localize it accurately with any of the existing methods. We are limited also in the case of a series of enzymes like the steroid dehydrogenases where, according to Marcus and Talalay (1956) the forward and backward velocities of the reaction are equal at pH 7·0.

Activation and Inhibition by Other Factors. In histochemical practice the effect of pH is not confined to altered kinetics of the enzyme reaction. As shown by Raaflaub (1953b) there is also an effect on the mitochondria. These are stable at pH 6·0 in isotonic solutions of non-electrolytes but at pH 7·4 and above very considerable swelling takes place. When this occurs the velocity of oxidation of various substrates is altered. Succinate is oxidized much faster by swollen mitochondria (Raaflaub, 1953a) but citrate, fumarate, α-ketoglutarate, malate and pyruvate are oxidized more slowly. This observation was confirmed histochemically by Scarpelli and Pearse (1958) who found that the *velocity* of the succinic dehydrogenase reaction, but not of the DPNH-diaphorase reaction, was markedly increased in swollen mitochondria. Slightly different results were obtained by Ziegler and Linnane (1958) who found that the oxidation of *iso*-citrate, aconitate, malate, lactate and β-hydroxybutyrate by ox heart mitochondria was in all cases increased following damage to the mitochondrial membrane.

As already noted in Chapter XIII, pp. 367–369, mitochondria can be protected against non-osmotic types of swelling by the addition of ATP. The mechanism by which this acts is not clear but Leuthardt and Bruttin (1952) have shown that it is not used up in the process. Ernster and Low (1955) and Raaflaub (1956) both suggest that ATP may function by chelating intramitochondrial calcium. It is now considered that mitochondrial damage leads to release of intramitochondrial calcium and that this impairs the process of oxidative phosphorylation. This in turn results in failure to synthesize ATP, with a consequent fall in intramitochondrial ATP leading to mitochondrial swelling.

Another factor which affects the stability of mitochondria is the presence or absence of Mg^{2+} ions, which can be replaced to some extent by Mn^{2+} or Co^{2+}. The mechanism by which magnesium ions exert their protective effect is at present unexplained although Ernster and Löw postulate that they bind ATP to the mitochondrion, thus preventing its loss. It appears to have been a fortunate accident that the best chelating ion (cobalt) for the monotetrazolium salt metal-chelation techniques should have been one which protected mitochondrial morphology.

The effect of the incubating medium on the mitochondria should

always be given the fullest consideration in designing reactions for dehydrogenase histochemistry. The use of hypertonic non-electrolyte media is absolutely essential for accurate localization of dehydrogenases and especially is this so in the case of soluble or partially soluble enzyme systems. The fullest possible protection is afforded by the use of 7·5 per cent. polyvinyl pyrrolidone and 0·005 M-magnesium ions in the incubating medium. Additional ATP is not usually required.

The Role of Coenzymes

The specific coenzymes act as carriers in the passage of electrons and hydrogen from the original substrate to molecular oxygen. Those with which we are here concerned are Coenzymes I and II, commonly called diphosphopyridine and triphosphopyridine nucleotides (DPN and TPN). They can be regarded firstly as coenzymes in the various dehydrogenase reactions and secondly as substrates for further enzyme systems. Using the alcohol dehydrogenase system once more as an example

$$\text{Alcohol} + \text{DPN}^+ \xrightleftharpoons{\text{dehydrogenase}} \text{Acetaldehyde} + \text{DPNH} + \text{H}^+$$

The coenzyme is readily and reversibly reduced by the dehydrogenase and the mechanism of this reduction has recently been elucidated by Fisher *et al.* (1953), using deuterium-labelled substrates. It had already been shown by Warburg and his associates (Warburg, Christian and Griese, 1935 ; Warburg and Christian, 1936) that the active part of the molecule was the nicotinamide moiety and that this underwent reversible reduction and oxidation. Fisher *et al.* showed that when deuterium-labelled alcohol was used the reduced coenzyme on the right of the equation contained deuterium, as did the acetaldehyde. If the deuterium-containing coenzyme was re-oxidized in a dehydrogenase-catalyzed system, as for instance by lactate dehydrogenase, the product (pyruvate) contained the deuterium. This shows that the process is, in fact, a direct transfer of hydrogen from substrate to coenzyme.

The reduced forms of Coenzymes I and II are not oxidized at significant rates by O_2, by dyes such as methylene blue, by tetrazolium salts or by cytochrome c. They are normally oxidized by cytochromes c or b_5 in the presence of their respective cytochrome reductases, and by methylene blue and tetrazolium salts in the presence of the appropriate diaphorase.

Diaphorases. An enzyme which catalyzed the oxidation of reduced Coenzyme I (DPNH) by methylene blue or cytochrome c. was independently discovered by Green, Dewan and Leloir (1937) and by von Euler and Hellström (1938). Shortly afterwards Straub and his co-workers succeeded in purifying the enzyme (Straub, 1939 ; Corran,

Green and Straub, 1939) which was characterized as a flavoprotein and referred to as DPN diaphorase (DPND). At the same time Adler *et al.* (1939) concluded that the oxidation of TPNH was carried out by a separate diaphorase since the oxidation of TPNH and DPNH by the tissues goes on at very different rates. Further investigations carried out by Racker (1955) have firmly established the part played by DPND and TPND in the electron transport system of the cell. According to Mahler (1955) DPND prepared from heart muscle by the method of Straub is a degraded form of DPNH cytochrome c reductase, differing from the latter in having lost its iron atoms and hence its capacity for reducing cytochrome c. A highly purified diaphorase produced by Savage (1957) was found to be completely inactive with cytochrome c as electron acceptor and it was impossible to convert it to the reductase by addition of Fe^{2+} or Fe^{3+} ions. Massey (1958a), however, still believes that the diaphorase is an undegraded enzyme.

From the practical point of view these arguments are not of much concern to the histochemist but if we are to interpret the results of studies on the two diaphorases it is essential to understand them. Further work by Massey (1958b) on the possible physiological role of DPND suggests that the function of the enzyme is to couple the oxidation of DPNH and the reduction of cytochrome c, employing a small molecular weight electron carrier as intermediate between the large molecules of cytochrome c and diaphorase. Out of a large number of redox systems tested by Massey α-lipoic acid was found to act as an efficient coupling agent. According to Massey the pH optimum of DPNH oxidation is 4·8 when ferricyanide is the electron acceptor, 5·9 when the acceptor is DL-lipoic acid and 6·5 with DL-lipoamide. The optimum with tetrazolium salts has not been determined. It seems reasonable to conclude that in demonstrating the level of diaphorase activity histochemically we are demonstrating the function of cytochrome c reductase as well, and also any recorded properties ascribed either to the reductase or to the diaphorase.

Histochemistry of the Diaphorases. The histochemical localization of DPND and TPND was first accomplished by Farber *et al.* (1956). These authors used an exogenous dehydrogenase together with its substrate and oxidized Coenzyme I (DPN) in order to generate DPNH as substrate for the intrinsic diaphorase of the tissues. They were able to demonstrate TPND without exogenous dehydrogenase, however, by supplying a substrate for endogenous dehydrogenases. Subsequently Nachlas and his co-workers (Nachlas, Walker and Seligman, 1958a) developed a method based on the same principle using Nitro-BT in place of the older BT or NT. This method provides an improved localization of diaphorase but does not demonstrate all sites of DPND activity unless exogenous enzyme is added. The method of Scarpelli,

Hess and Pearse (1958), employing DPNH and TPNH as substrate, is described in Chapter XX and Appendix 20. It does not require exogenous dehydrogenase for the optimal demonstration of diaphorase activity and thus avoids a complication due to spontaneous reduction of tetrazolium salt in the incubating medium when the exogenous dehydrogenase is contaminated with diaphorase. Racker (1955) has shown that this is the case with alcohol dehydrogenase.

The very considerable differences observed in the rate of tetrazolium reduction and cellular localization of diaphorase activity when TPNH is substituted for DPNH suggest that the oxidation of Coenzymes I and II is certainly mediated by separate enzymes.

Further Considerations. The reduced coenzyme can certainly be considered as substrate for the diaphorase :

$$\text{DPN (TPN)H} + \text{tetrazole} \rightleftharpoons \text{DPN(TPN)}^{+} + \text{Formazan}$$

Since the reaction as it stands is not reversible the equilibrium should lie to the right (formazans are not re-oxidized to tetrazoles in biological systems). The oxidation of DPNH or TPNH by diaphorase should not therefore be a limiting factor in deciding the overall rate of a pyridine nucleotide-linked dehydrogenase system in histochemical practice. If, however, the DPN^{+} is removed by some process (see below) the supply must be maintained either by the presence of sufficient DPNH (diaphorase reaction) or of sufficient DPN for conversion to DPNH (dehydrogenase reactions).

Arguments for Use of High Coenzyme Levels

Despite the apparent preservation of mitochondrial morphology achieved by the use of protective media the freezing and thawing of the normal cold microtome (cryostat) procedure results in damage to the mitochondrial membrane and in the release of soluble co-factors (Porter *et al.*, 1953). Endogenous coenzyme levels are too low to allow histochemical dehydrogenase reactions (coenzyme-dependent) to take place at all and exogenous DPN or TPN must be added. Hess, Scarpelli and Pearse (1958) found that the optimal reduction of tetrazolium salt occurred when the co-enzyme was present in the same concentration as the substrate (0·1M) although good results were obtained by using one-tenth of this amount (0·1M, or 6 mg./ml.). These figures are much higher than those used by other workers. For example Nachlas, Walker and Seligman (1958a and b) used 0·5 mg./ml. of DPN and 0·3 mg./ml. of TPN in their methods for DPN and TPN-linked enzyme systems.

There are many reasons why high coenzyme levels should be maintained in histochemical media and many factors operate under histochemical conditions to reduce the amount of available DPN or

TPN to a low level. Some of these are of serious concern if incubation times are prolonged.

The binding of coenzyme by the dehydrogenase (Straub, 1940) does not normally lead to deficiency because the enzyme is present in relatively low concentration. Coenzyme-linked dehydrogenases can be divided into "alcohol and aldehyde" varieties and fundamental differences exist between these two classes. Those which can be classified as "alcohol" dehydrogenases oxidize primary alcohols to aldehydes, secondary alcohols to ketones, primary amines to ketones and ammonia and hemi-acetals to lactones. Examples in these subgroups are alcohol dehydrogenase, lactic dehydrogenase, glutamic dehydrogenase and glucose-6-phosphate dehydrogenase. San Pietro and his associates (van Eys, San Pietro and Kaplan, 1958), however, have shown that the mechanism of action of DPN and TPN-dependent dehydrogenases requires the binding of two moles of DPN per molecule of enzyme. This is shown below in Fig. 181 (I) where the enzyme is represented by a hatched bar with two sulphydryl atoms, each of which binds a molecule of the coenzyme.

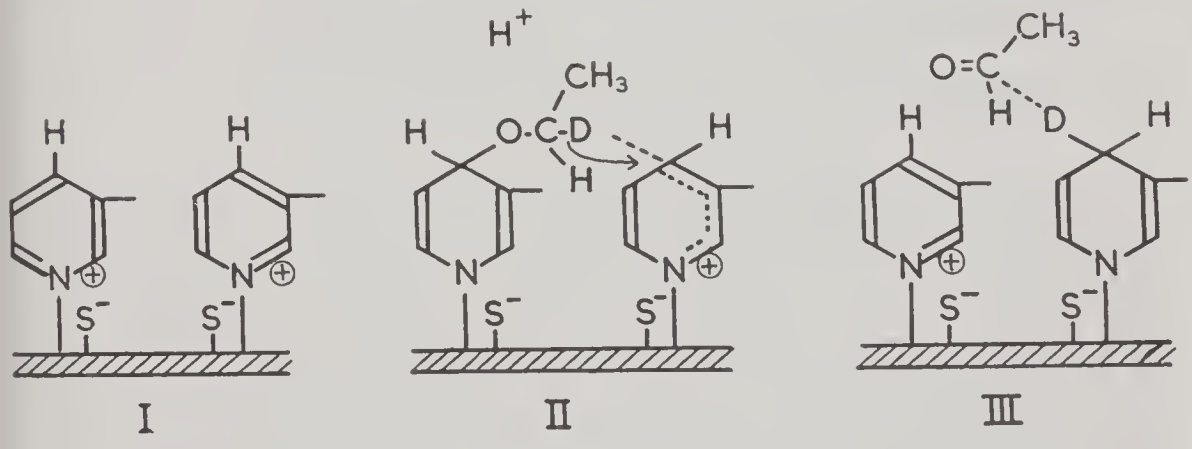


Fig. 181.

These diagrams represent the following equations :

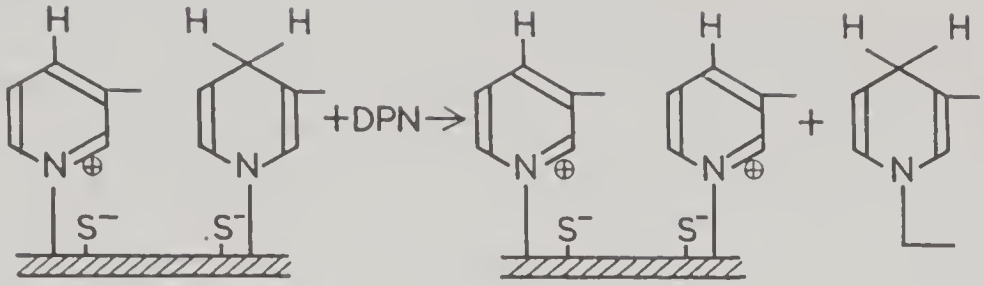
$$E + DPN + DPN \longrightarrow E\text{-}DPN_2 \text{ (I)}$$

$$E\text{-}DPN_2 + S \longrightarrow E\text{-}DPN\text{-}DPNS + H^+ \text{ (II)}$$

$$E\text{-}DPN\text{-}DPNS \longrightarrow E\text{-}DPN\text{-}DPNH + P \text{ (III)}$$

We see that the enzyme binds two molecules of coenzyme and is then able to activate the substrate, deutero-ethanol in the diagrams in Fig. 181, with loss of one hydrogen atom and the transfer of the other to the second coenzyme molecule. After release of the product (P) the

enzyme-coenzyme complex is thus partly reduced. It is regenerated as follows :



IV

Fig. 182.

This diagram (Fig. 182) represents the following equation,

$$\text{E-DPN-DPNH} + \text{DPN} \longrightarrow \text{E-DPN}_2 + \text{DPNH} \quad \text{(IV)}$$

which is the normal process by which the enzyme-coenzyme is oxidized to its original state. Two further reactions may occur and these can be written :

$$\text{E-DPN-DPNH} + \text{S} \longrightarrow \text{E-DPNS-DPNH} + \text{H}^+ \quad \text{(V)}$$

and $$\text{E-DPN-DPNS} + \text{S} \longrightarrow \text{E-DPNS}_2 + \text{H}^+ \quad \text{(VI)}$$

Both these reactions will result in inactivation of the enzyme and competition will occur between reactions IV and V and also between reactions III and VI. It is evident from the above equations that, even if DPN were not destroyed in the tissues but regenerated *in toto* for further use in reaction IV, it would still be necessary to maintain a high concentration of coenzyme in histochemical media in order to satisfy the kinetics of reactions I and IV. From reactions I and II it is clear that *the affinity for the substrate depends on the DPN concentration.*

Factors causing Destruction of DPN and TPN. At acid pH levels DPN is quite stable (Kaplan, Colowick and Carr Barnes (1951) and it can conveniently be stored in solution at $-20°$. At alkaline pH levels, on the other hand, its destruction is rapid. The alkaline pH levels at which many workers carry out their dehydrogenase reactions must be condemned for this reason as well as for others. A neutral or slightly acid pH is preferable and this has the additional effect of maintaining mitochondrial structure (Scarpelli and Pearse, 1958).

The reduced coenzymes (DPNH and TPNH) show the opposite characteristics, being unstable in acid but relatively stable in alkaline solutions. The marked inhibitory effect of high hydrogen ion concentration on the oxidation of TPNH by its diaphorase may well be

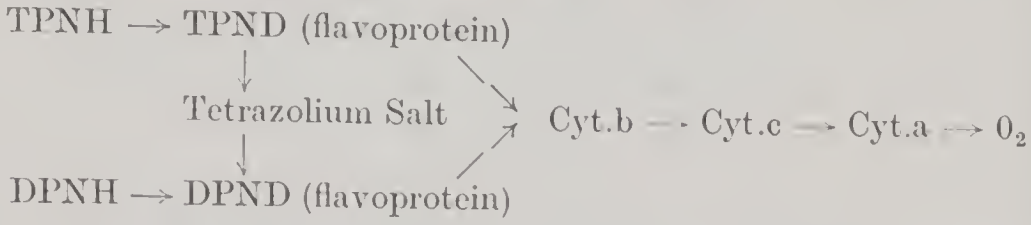
due to isomeric conversion of the adenylic acid ribose moicty of TPNH since Shuster and Kaplan (1955) have shown that the monophosphate group of adenylic acid ribose migrates from the 2′ to the 3′ position at acid pH levels and the 3′-TPN which is formed is incapable of functioning with enzymes that are strictly specific for the naturally occurring 2′-TPN. The whole subject is ably reviewed by Singer and Kearney (1954).

The only other important factor which concerns the coenzymes in histochemical practice is their destruction by nucleotidases. This cannot be prevented though attempts have been made to reduce it by adding a competitive inhibitor, nicotinamide, to the incubating medium (Kochakian *et al.*, 1957). Under histochemical conditions this substance appears to exert little effect and far better results are achieved by maintaining high coenzyme levels in the incubating medium, despite the expense involved in this procedure.

Effect of High DPN Levels at Alkaline pH. Provided that the pH of the incubating medium is maintained at 7·0 or below no untoward circumstances are observed in tissue sections. Above pH 7·0, however, an effect occurs which may be referred to as " nothing dehydrogenase " using the loose but descriptive term employed by Racker (1955). It was first noted by Hopkins and Dixon (1922) that well-washed tissue residue possessed marked reducing capacity and Racker and his associates found that well-dialysed protein fractions obtained from various tissues could catalyse the reduction of DPN or TPN in the absence of any substrate. They assumed that this activity was connected with the presence of sulphydryl groups. Zimmermann and Pearse (1959), while endeavouring to demonstrate a specific sorbitol dehydrogenase, observed with both Nitro-BT and MTT the production of formazan in systems containing DPN (or TPN), buffer and tissue section only. This effect (Fig. 183, p. 560) increased progressively from pH 7·0 up to pH 9·0 and decreased thereafter. It was not demonstrable at pH 6·5. " Nothing dehydrogenase " was inactivated by a number of sulphydryl inhibitors, incorporated in the incubating medium at a concentration of 0·1M. Among those tested were N-ethyl maleimide, iodoacetate and *p*-chlormercuribenzoic acid. Preincubation of sections with 0·1M-N-ethyl maleimide for one hour at 37° also completely abolished the reaction. The substitution of DPNH for DPN produced a positive reaction in the presence of any of the inhibitors, indicating lack of interference with diaphorase activity. Other considerations make it likely that protein-bound SH is responsible but the participation of smaller molecules like glutathione has not been excluded. The reaction should be useful as a demonstrator of " active sulphydryl " groups, if it can be shown that the localization of the formazan is not the general localization of diaphorase.

Blocking of the Preferential Electron Pathway

The Respiratory Chain. As previously emphasized, all dehydrogenase reactions at present susceptible to histochemical demonstration depend on the transfer of electrons via coenzyme and diaphorase, with the sole important exception of succinic dehydrogenase. The respiratory chain involved in the oxidation of reduced coenzymes can be represented (incompletely) as follows :



The natural tendency, provided that all the factors in the respiratory chain are present and functioning normally is for electrons to be transferred successively through the various stages of the chain to molecular oxygen. The whole scheme has been referred to by Lundegardh (1958) as the electron ladder, though he illustrates the passage of electrons as a stream passing over a series of waterfalls, since successive stages involve a progressive rise in redox potential.

It will be realized that the dehydrogenase is the first member of a chain of enzymes which transfer H atoms or electrons from the substrate to molecular oxygen. An oxidase, on the other hand, is an enzyme whose hydrogen acceptor is molecular oxygen. It is usually named after the hydrogen donor.

In histochemical practice there are two principal ways in which attempts are made to channel the flow of electrons into the tetrazolium trap, so that the maximum amount of formazan is produced. The first is by blocking the electron chain so that the maximum amount of formazan is produced, and the second is by the use of alternative electron acceptors which are capable of reversible oxidation by tetrazolium salts.

The electron pathway has been the subject of a large number of diagrammatic representations. The one reproduced in Fig. 184, below, shows the salient features of the chain as it concerns the histochemist. In this diagram the various blocking agents appear in squares and the points at which exogenous acceptors are able to take up hydrogen atoms and electrons are indicated by circles.

Terminal Chain Block. The classical method by which the respiratory chain has been blocked, in histochemical practice, has been by carrying out the various reactions under anaerobic conditions. As an alternative, the cytochrome oxidase part of the system has been blocked with cyanide. Both these procedures usually result in increased production of formazan and, in many cases, one or the other

FIG. 177. Rat kidney. Succinate dehydrogenase reaction leading to production of formazan from MTT. Finely crystalline at first, crystal growth occurs rapidly, leading to the patterns shown. × 300.

FIG. 178. Rat kidney. As Fig. 177, but incubated in presence of cobaltous ions (0·0001 M). × 900.

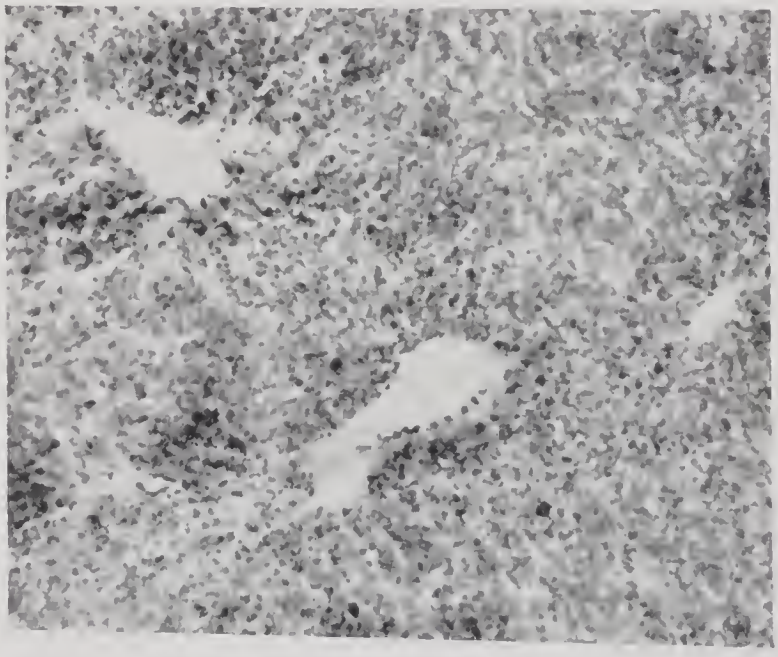


FIG. 183. Rat liver. Deposits of formazan throughout the liver lobule indicate strong activity of "nothing dehydrogenase" at pH 9·0. Nitro-BT. × 470.

[*To face p.* 560.

FIG. 187. Rat kidney. Cold microtome section. Shows intramitochoudrial activity of glucose-6-phosphate dehydrogenase in the cells of the macula densa. MTT-cobalt method. × 1,400.

FIG. 188. Rat kidney. Cold microtome section. Shows intramitochondrial localization of β-hydroxybutyrate dehydrogenase in cells of the descending loop of Henle. MTT-cobalt method. × 4,400.

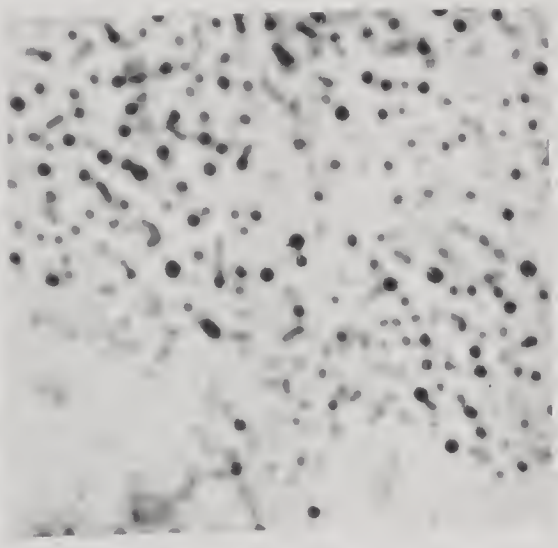


FIG. 189. Rabbit. Spinal ganglion cell. Shows effect of over-incubation on intramitochondrial formazan deposits (succinate dehydrogenase reaction). MTT-cobalt method. × 2,500.

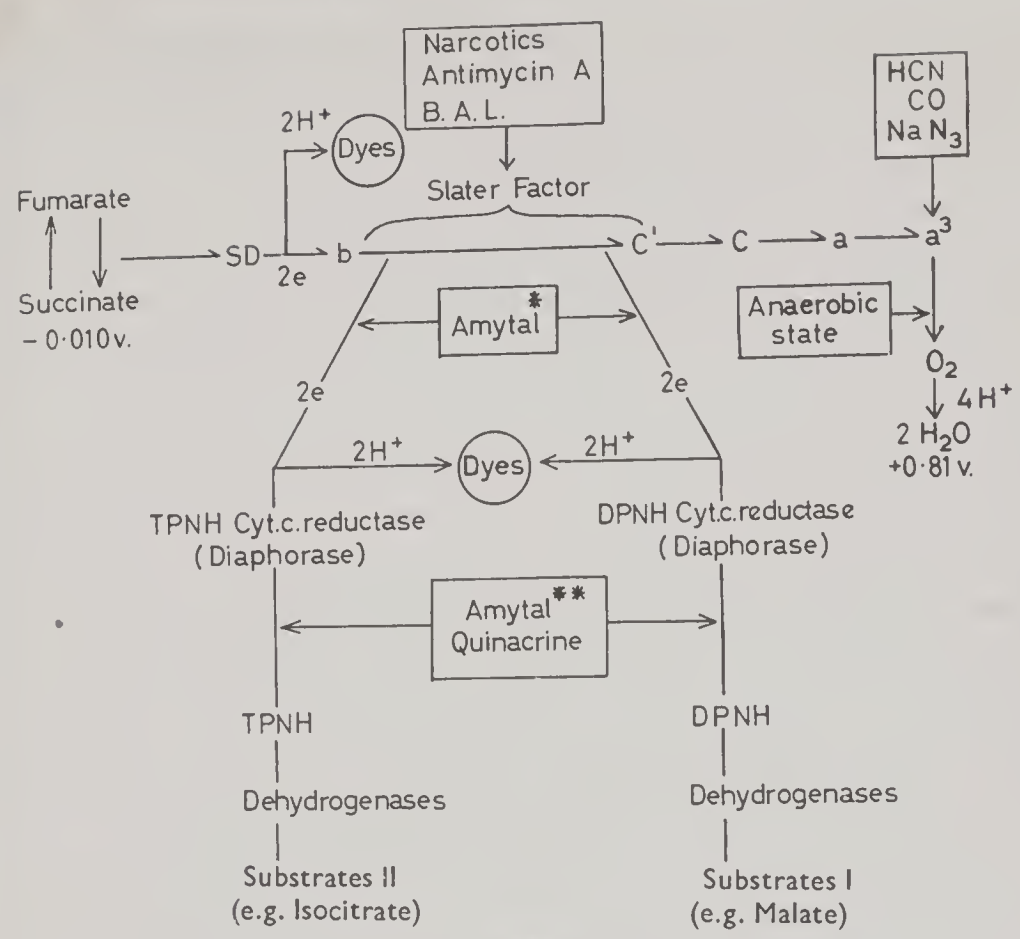


FIG. 184. Electron Pathway (After Hartree, 1957).

* Probable site in frozen-thawed mitochondria.
** Probable site in normal mitochondria.

manœuvre has been essential. Azide, though less frequently employed, has an effect similar to but often better than cyanide. In the case of the DPND reaction (Chapter XX, p. 570) only a slight increase in the rate of reduction of tetrazolium is observed when incubation is carried out in an atmosphere of nitrogen. Azide, however, shows a considerable effect (cyanide cannot be employed with the MTT-cobalt system). In the techniques for specific dehydrogenases using Nitro-BT as acceptor, cyanide was found by Hess, Scarpelli and Pearse (1958) to be an efficient respiratory inhibitor. Absence of oxygen was neither effective nor necessary.

Mid-chain Block. Reference to Fig. 184 shows that a number of respiratory inhibitors have been described which act at points in the respiratory chain on the oxygen side of the flavoprotein carrier, whatever the identity of the latter. In particular, the work of Ernster, Jalling, Löw and Lindberg (1955) and of Jalling, Lindberg and Ernster (1955) suggested that amytal was a most efficient inhibitor for all DPN-linked systems. It was found that 1·8 mM-amytal completely blocked all DPN-linked oxidation, leaving succinate oxidation completely unaffected. Out of a large number of compounds tested

Scarpelli, Hess and Pearse (1958) found that amytal was the most efficient, increasing the rate of reduction of the tetrazolium salt in the DPN diaphorase reaction so that 5 minutes' incubation was adequate. Azide was only slightly less effective.

Although according to Chance (Chance and Williams, 1956; Chance, 1956) amytal was observed to block electron transfer in liver mitochondria at a point between DPNH and flavoprotein (i.e. at the site marked ** in Fig. 184) the rapid rate of tetrazolium reduction by tissues in the presence of reduced pyridine nucleotide and amytal suggests that the site of amytal effect in tissue sections is beyond flavoprotein (* in Fig. 184). Recently Estabrook (1957) has shown a similar site of amytal inhibition in the case of the DPNH cytochrome c reductase system and he suggested that the difference between these findings and those of Chance might indicate structural alteration of the electron transfer chain due to damage to the mitochondria during preparation. The results obtained by Chance and Baltscheffsky (1958), in heart muscle sarcosomes have confirmed the original findings made on liver mitochondria, that the site of amytal block is between DPNH and flavoprotein. Electron transport from non-DPN-linked substrates, such as succinate, was not found to be inhibited by amytal. The results obtained histochemically may possibly be explained on similar lines to those put forward by Estabrook since freezing and thawing are known to damage the mitochondrial membrane. The structural labilization of mitochondria by pre-treatment with Ca^{2+} or in the absence of Mg^{2+}, leading to decreased sensitivity to amytal blocking of respiration, has been extensively discussed by Ernster (1956). It remains a fact, however, that in fresh smears, incubated without being allowed to dry, amytal fails to block diaphorase or pyridine nucleotide-linked dehydrogenase reactions. Instead it produces the same augmentation of formazan production. Fresh tissue smears are admirable objects for study; an effective technique for making them has been described by Branster and Morton (1957).

Competitive Electron Acceptors. The results obtained by Baltscheffsky (1957) add additional support to the histochemical findings reported above. Using the DPNH oxidase system this author found that the inhibition of respiration (measured by 0_2 uptake) by 2×10^{-3} M-amytal or $2{\cdot}4 \times 10^{-6}$M-antimycin could be offset, though not entirely reversed, by the addition to the medium of 10^{-5}M-cytochrome c. This suggests that the site of amytal action is beyond flavoprotein and that it probably involves cytochrome c. Baltscheffsky's results are shown in Fig. 185, below. The Baltscheffsky effect can be demonstrated histochemically in the DPNH diaphorase reaction though, of course, the reverse effect is found. That is to say, the increased formazan production brought about by effective amytal blocking of the respiratory

chain below the point at which electrons pass to the tetrazolium salt is offset by the alternative electron pathway provided by the exogenous cytochrome c. That such pathways can be produced is demonstrated by the results recorded above. Whether they exist *in vivo* or in tissue preparations is a question that requires an answer. Certainly it seems possible that naturally-occurring substances like quinones (e.g. ubiquinone ; Morton *et al.*, 1958 ; Fahmy *et al.*, 1958) can transfer electrons

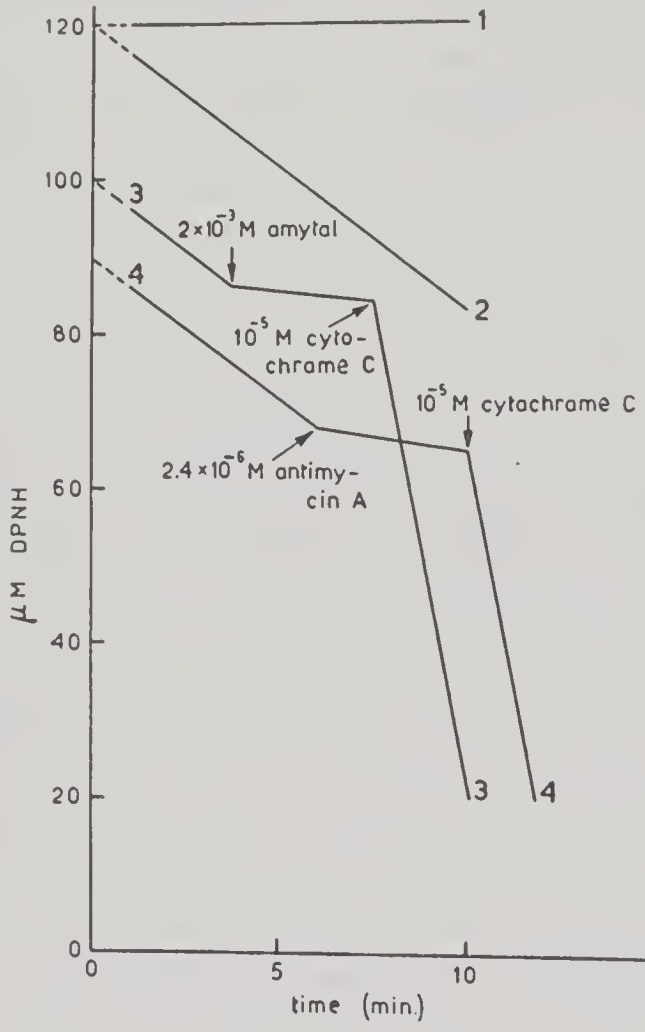


FIG. 185. Effect of Amytal and Antimycin on DPNH oxidase.

from the flavoproteins to cytochrome c, by-passing intermediate stages of the respiratory chain. If such substances are present in tissue preparations electrons may escape by this path. Combined mid-chain and terminal chain block, as employed in systems using Nitro-BT, amytal, and cyanide as trapping agent, would be expected to overcome any escape of electrons induced by mechanisms of this type. It seems likely, therefore, that unknown pathways exist which prevent us from approaching the desired figure of 100 per cent. efficiency for the trapping of electrons by the tetrazolium salt.

Other Alternative Electron Pathways. The use of soluble redox

dyes to act as intermediate acceptors between diaphorases and tetrazolium salts was introduced by Farber and Louviere (1956) who employed methylene blue (redox potential 0·011v.), together with the relatively inefficient Blue tetrazolium salt (redox potential — 0·17v.) which was in general use at that time. Several other dyes were found to enhance formazan production. Among these were thionin (+0·06v.) and pyocyanin (—0·034v.). Another suggestion made by Farber and Louviere followed the observation by Singer and Kearney (1954a) that phenazine methosulphate could reversibly accept electrons directly from succinic dehydrogenase. Later Singer, Kearney and Massey (1957) suggested that phenazine (and ferricyanide) could accept electrons directly from the Fe^{2+} ions of the iron-flavoprotein whereas the other dyes, including methylene blue and tetrazolium salts, could accept only from the flavin part of the molecule. Dyes of this variety are thus relatively inefficient and Singer believes that the natural acceptor can take electrons from Fe^{2+}, as does phenazine.

Personally I was never able to produce any improvement in the diaphorase reaction by the use of soluble redox dyes like methylene blue and thionin and these are in any case unnecessary now that tetrazolium salts with higher redox potentials are available for histochemical use. The findings of Nachlas *et al.* (1958) confirm these observations and these authors have suggested that one would not expect any response from redox dyes when using the more favourable tetrazolium salts. This is not necessarily true in the light of Singer's observations and I have found that phenazine methosulphate is initially able to speed up the velocity of the succinic dehydrogenase and diaphorase reactions, using Nitro-BT or MTT. Later inhibition (possibly by breakdown products) occurs and the reaction is slowed and stopped.

Phenazine methosulphate is the only redox dye whose use should be considered in modern histochemistry. It may permit one to demonstrate the presence of otherwise undemonstrable activities (e.g. choline dehydrogenase) though the exact mechanism through which it operates remains uncertain. These considerations bring one to the final point of this chapter.

Redox Potentials of Oxidative Step and Acceptor. The relationship between the redox potential of the acceptor (tetrazolium salt) and that of the stage in the respiratory chain (or other reaction) at which the electrons are acquired, merits brief consideration. The redox potential of the enzyme-bound DPN-DPNH system was found by Theorell and Bonnichsen (1951) to be —0·21v. and DPNH can be oxidized by the cytochrome b5 of liver microsomes (—0·12v.). According to Burton and Wilson (1953) the E° of TPN is —0·324v. at pH 7·0 and 25°. Since the redox potential of the newer tetrazoles is even higher than this

there is no *a priori* reason why electrons should not flow from DPNH to these compounds, as they do in practice through the medium of the diaphorase. The DPN-linked lipoic acid dehydrogenase system has a very low potential, −0·42v. according to Reed (1953), and Massey (1958b) has recently shown that a diaphorase extracted from pig heart muscle behaves as a powerful lipoic acid dehydrogenase, as mentioned above. Almost all the electron acceptors used in dehydrogenase histochemistry have potentials higher than this.

We are thus confronted with no essential difficulty from the histochemical point of view provided that our tetrazolium salt has a higher redox potential than the system from which it is to accept electrons, and provided that the overall kinetics of the reaction can be adjusted to induce the reaction to proceed to the right.

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CHAPTER XX

DIAPHORASES AND DEHYDROGENASES

Historical Introduction

THE first stage of dehydrogenase histochemistry began, as stated in Chapter XIX, with the use of monotetrazolium salts as electron acceptors for known or unknown dehydrogenase systems. These salts (TTC and INT) were relatively useless from the histochemical point of view, providing a poorly coloured diffusible end product in one case and a macrocrystalline one in the other. During the second stage of dehydrogenase histochemistry a great expansion of ideas took place when the techniques of the science were improved by the advent of Neotetrazolium (NT) and Blue tetrazolium (BT). Almost all the applied dehydrogenase histochemistry reported in the literature between 1950 and 1958 has been carried out with one or other of these two salts.

For those who have neither the time nor the inclination to digest the subject matter of Chapter XIX it may be sufficient here to state that with the advent in 1957 of new tetrazolium salts, and new principles for applying them, the whole face of dehydrogenase histochemistry has been changed. In place of relatively crude localization at the histological level we now have intramitochondrial localization and in place of a single dehydrogenase and two so-called diaphorases we can now demonstrate a spectrum of specific dehydrogenases. This is no minor change but a revolution and it must be appreciated, therefore, that much of the pioneer work in the dehydrogenase field now requires revision. Nevertheless a list of such work (necessarily incomplete) is given here since it forms a base line from which new studies in dehydrogenase histochemistry must advance.

Applied Dehydrogenase Histochemistry

General Studies and Reviews. Seligman and Rutenburg (1951); Shelton and Schneider (1952); Padykula (1952); Rutenburg, Wolman and Seligman (1953); Malaty and Bourne (1953); Rosa and Velardo (1954); Novikoff (1955); Baar (1957); Ogawa (1958). *Central Nervous System and Ganglia.* Leduc and Wisłocki (1952); Mustakallio (1954a); Ortmann (1957); Blasius and Zimmermann (1958). *Neoplastic Tissues.* Goddard and Seligman (1953); Hsu and Hoch-Ligeti (1953); Pearson and Defendi (1955). *Thyroid.* Goddard and Seligman (1952); Kuusisto and Telkkä (1955). *Kidney.*

Mustakallio and Telkkä (1953) ; Bourne and Malaty (1953) ; Wachstein and Meisel (1954) ; Wachstein (1955) ; Telkkä and Mustakallio (1955) ; Mustakallio and Telkkä (1955) ; Reale and Luzzatto (1956) ; Pearse and Macpherson (1958) ; Rudolph and Scholl (1958). *Embryos.* Rossi, Pescetto and Reale (1954, 1957). *Liver and Gall-Bladder.* Mustakallio (1954b) ; Mustakallio and Saikkonen (1955) ; Schumacher (1957). *Skin and Appendages.* Rogers (1953) ; Formisano and Montagna (1954) ; Montagna and Formisano (1955) ; Portugalov (1955) ; Mustakallio (1956) ; Argyris (1956a and b) ; Cruickshank, Hershey and Lewis (1958). *Cells in Blood or Tissue Culture.* Hughes (1956) ; Marcuse (1957a and b) ; Vercauteren (1957). *Blood Vessels.* Fried and Zweifach (1955). *Muscle.* Malaty and Bourne (1953) ; Beckett and Bourne (1958) ; Nachmias and Padykula (1958) ; Buno and Germino (1958). *Heart Muscle.* Bourne (1953) ; Cooper (1955) ; Wachstein and Meisel (1955) ; Cooper and Copenhaver (1953). *Female Genitalia.* Foraker, Denham and Johnston (1953) ; Foraker and Denham (1953) ; Rosa and Velardo (1958). *Bacteria.* Davis *et al.* (1953) ; Mustakallio and Jännes (1954) ; Davis and Mudd (1957).

Further Advances in Dehydrogenase Histochemistry

The end of the second stage of development was marked by the pioneer work of Farber and his associates (Farber, Sternberg and Dunlap, 1956), who used NT and BT as their hydrogen acceptors. Farber developed the thesis that three systems only could be demonstrated by the use of tetrazolium salts. These were succinic dehydrogenase, DPN diaphorase and TPN diaphorase. This argument was supported by the observation that whatever the substrate employed the localization of formazan differed only when TPN was substituted for DPN, with an enzyme-substrate system capable of employing it as coenzyme.

The techniques developed by Farber were not entirely reliable in the hands of other workers and the results, though æsthetically attractive (Plate VIId, p. 545), were difficult to interpret. Farber himself stated that they were satisfactory for *histochemical* purposes only and that it could not be demonstrated that the formazan deposits accurately indicated the intracellular localization of enzyme activity. Although occasional workers claimed that they were showing the presence of one or other of the specific dehydrogenases the majority accepted Farber's dictum that whatever the substrate the histochemical demonstration of a pyridine nucleotide-linked dehydrogenase system afforded only a localization of the appropriate diaphorase. It was clearly demonstrated, as mentioned above, that DPN and TPN diaphorases have entirely different localization in mammalian tissues.

Methods for DPN and TPN Diaphorases (DW. 242, 243)

These two flavoprotein enzymes transfer hydrogen and electrons from the reduced coenzymes (DPNH, TPNH) to the tetrazolium salt and no intermediate acceptor is required. In the original method (Farber *et al.*, 1956a) both were demonstrated by maintaining a supply of reduced coenzyme either through the activity of an exogenous enzyme-substrate system which was added to the medium, together with the oxidized coenzyme (DPN or TPN), or through the activity of an endogenous dehydrogenase whose substrate was provided. For DPN diaphorase, using endogenous enzyme, malate and glutamate were added but better results were obtained when ethanol and exogenous alcohol dehydrogenase were employed. In the case of TPN diaphorase an exogenous enzyme was not used. Instead, either glucose-6-phosphate or a mixture of citrate and malate were added as substrates for endogenous enzymes. The addition of cysteine or thioglycollate was found essential and the presence of a sulphydryl compound was regarded as an absolute requirement for the three TPN-linked enzymes studied by Farber and his coworkers. At the pH employed cysteine and thioglycollate can produce non-enzymic reduction even of tetrazolium salts with relatively low redox potentials like NT and BT. They must never be used with tetrazolium salts having higher potentials, like MTT and Nitro-BT.

With the advent of Nitro-BT a great improvement in the localization of the diaphorases became possible and Nachlas, Walker and Seligman (1958a) demonstrated DPN diaphorase with this salt and a system containing lactate and lactic dehydrogenase at pH 7·4. Later (1958b) these authors applied their findings to the demonstration of TPN diaphorase. They considered two alternative procedures : 1) the inclusion in the medium of two substrates for endogenous TPN-linked dehydrogenases and 2), the addition of an exogenous TPN-linked dehydrogenase and its substrate (*iso*citrate and *iso*citrate dehydrogenase). Since this enzyme was not easily available the first alternative was employed. Experiments were performed with four substrates in various combinations. The mixtures which resulted in a summation of enzymic activity were *iso*citrate plus malate, *iso*citrate plus glucose-6-phosphate and 6-phosphogluconate plus malate. An unexplained but understandable finding was the failure to note summation with the combination of glucose-6-phosphate with malate and of glucose-6-phosphate plus 6-phosphogluconate. The *iso*citrate-malate combination was recommended as giving the best results. The localization of TPN diaphorase afforded by the above techniques appeared to the authors to be non-mitochondrial in contrast to the mitochondrial localization which they obtained with DPN diaphorase.

Use of Reduced Coenzymes as Substrates

A more simple and more effective procedure than the manœuvres described above is the use of DPNH and TPNH as substrates for their respective diaphorases (Scarpelli, Hess and Pearse, 1958). The reactions involve direct transfer of electrons and hydrogen from the reduced pyridine nucleotide to the diaphorase which in turn transfers them to the tetrazolium salt, reducing it to an insoluble formazan. Since neither reaction requires the addition of exogenous dehydrogenase, or substrate for endogenous systems, the techniques offer distinct advantages over those previously described. Details are given in Appendix 20, p. 908, together with the methods of Nachlas *et al.* The latter can be employed if, for any reason, reduced coenzymes or MTT are not obtainable.

The use of respiratory inhibitors (mid-chain block) has been fully described in Chapter XIX and it is sufficient here to state that the rate of reduction of tetrazolium salt in both DPN and TPN diaphorase systems is most effectively stimulated by the addition of amytal to the medium. The latter is easily prepared and can be stored in the cold for long periods.

The DPN and TPN diaphorase reactions with DPNH and TPNH as substrates demonstrate all sites of diaphorase activity. In both cases the localization of formazan is predominantly intramitochondrial although there is sometimes a faint non-particulate extramitochondrial distribution of pigment, with both systems. The reasons for this are not entirely clear but a possible explanation in the case of microsomal DPNH-cytochrome c reductase and the corresponding diaphorase comes from the work of Garfinkel (1957). This author has demonstrated the effect of metal ions as inhibitors of these enzymes. Cobalt ions at a concentration of 7×10^{-6}M were found to inhibit 95 per cent. of the diaphorase activity but only 19 per cent. of that of the reductase.

The DPN and TPN diaphorase reactions can be used to estimate the overall activity of a given tissue in respect of DPN or TPN-linked dehydrogenase systems (see Table 47, p. 578). They can also be used, and this applies particularly to DPN diaphorase, as mitochondrial indicators in place of the conventional cytological stains for these organelles. The mitochondria of some cells, however, fail to oxidize DPNH or TPNH even after long incubation periods. This may mean either that such mitochondria do not contain diaphorase systems or that the N-thiazolyl monotetrazolium salt employed (MTT) is insufficiently sensitive to demonstrate sites of low enzyme activity. The latter explanation is possible, but unlikely.

Succinate Dehydrogenase and the Specific Coenzyme-linked Dehydrogenases

List of Available Methods

The only dehydrogenase histochemically demonstrable was, until recent years, succinate dehydrogenase. Even then many workers were cautious in interpreting their results, when succinate was used as substrate, as being due to this enzyme. They preferred to evade the issue by using the term succinic dehydrogenase or succinic oxidase *system*. There is no present reason for supposing that modern tetrazolium salts with high redox potentials are not able to accept electrons from the built-in flavoprotein which is part of the succinate dehydrogenase complex. We are now able to demonstrate histochemically ten, and possibly eleven, of the specific coenzyme-linked dehydrogenases in addition to succinate dehydrogenase and the two diaphorases. Table 46, below, is a list of these enzymes divided into those which can be shown by means of the monotetrazolium salt-cobalt chelation techniques and those which must be shown by the best available ditetrazolium method.

TABLE 46

Methods for Dehydrogenases and Diaphorases

MTT-Co Method	DW No.	Nitro-BT Method	DW No.
DPNH Diaphorase	242 (348)	Alcohol Dehydrogenase	260
TPNH Diaphorase	243	Lactate Dehydrogenase	288
Succinate Dehydrogenase	338	*Iso*citrate Dehydrogenase (DPN)	305
Glucose-6-phosphate Dehydrogenase	312	*Iso*citrate Dehydrogenase (TPN)	304
6-Phosphogluconate Dehydrogenase	307	Glutamate Dehydrogenase	227 228
		α-Glycerophosphate Dehydrogenase	267
β-Hydroxybutyrate Dehydrogenase	292 (295)	Malate Dehydrogenase (TPN)	298
		Malate Dehydrogenase (DPN)	299

I have endeavoured, in another place (Pearse, 1958), to express the significance to cytologists, histologists and pathologists of our ability to show this wide spectrum of specific dehydrogenases. This we can do by means of methods having a high degree of sensitivity, which are capable of accurate localization, and with which it is probable that the amount of formazan deposited is directly related to enzyme activity. We can now see and record small alterations in the level of the preferential oxidation pathway in single cells and we can thus detect changes of cellular function such as that from normal growth to

neoplasia. In Fig. 186, below, the dehydrogenases whose activities we can at present demonstrate are shown by broad arrows. Stages which are marked by thin arrows in the Krebs' cycle, the pentose cycle (hexose monophosphate shunt), and in other oxidative pathways, cannot yet be demonstrated histochemically.

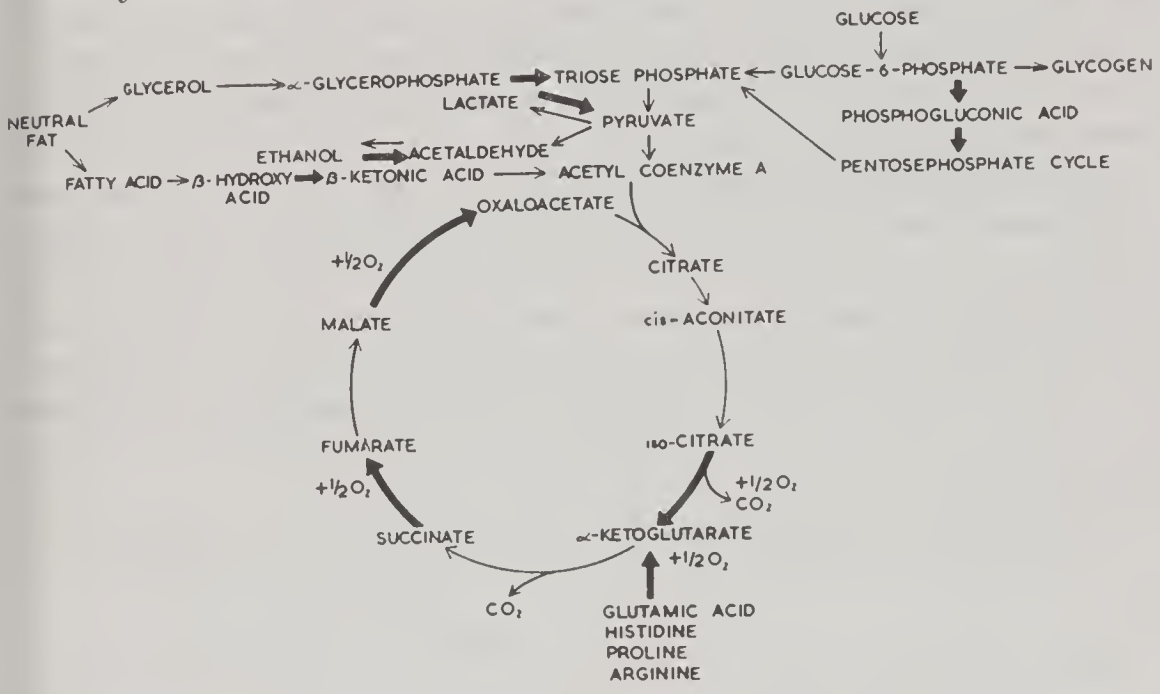


FIG. 186. Histochemically Demonstrable Dehydrogenases.

Histochemistry of Oxidative Pathways

The Krebs' Cycle. It was Szent-Gyorgyi who first suggested that succinate and fumarate acted as a carrier system for hydrogen removed in the aerobic oxidation of carbohydrate. Later, he discovered that malate and oxaloacetate could act in the same way. Unfortunately, when the whole scheme was tested with isolated components by Green and his associates the succinate system failed to couple with the DPN-linked malate system.

Resolution of this conundrum came when Krebs discovered the part played by α-ketoglutarate and citrate in the aerobic respiration of muscle and when, finally, he synthesized the whole chain into the cycle which bears his name by the finding that pyruvate and oxaloacetate could react to form a 7-carbon compound from which citrate and α-ketoglutarate could be formed once more.

Krebs' Cycle Enzymes. It can be seen from Fig. 186 that three of the dehydrogenase systems which we can demonstrate are main components of the tricarboxylic acid cycle. These are succinate, malate and *iso*citrate dehydrogenases. The latter is the TPN-linked enzyme which is found, in homogenization studies, mainly in the supernatant. The DPN-linked *iso*citrate dehydrogenase, on the contrary, is regarded as a mitochondrial enzyme. If protected mito-

chondria are employed, either isolated or in tissue sections, both enzymes are shown by modern histochemical techniques to be intramitochondrial.

Any or all of the three above-mentioned dehydrogenases can be used as histochemical indicators of activity of the Krebs' cycle though *iso*citrate dehydrogenase may provide α-ketoglutarate for use in synthetic systems outside the cycle. Succinate and malate dehydrogenases are thus the best indicators of Krebs' cycle activity from the histochemical point of view.

Glutamate Dehydrogenases. These enzymes have a very different significance from the foregoing enzymes of the Krebs' cycle. When coupled with transaminases (not demonstrable histochemically) they can synthesize a variety of amino-acids from keto-acids and ammonia. The glutamate in this case acts as an ammonia carrier. Working in conjunction with *iso*citrate dehydrogenase and Coenzyme II, glutamate dehydrogenase can use up α-ketoglutarate provided by activity of the first enzyme.

$$\textit{Iso}\text{citrate} \xrightarrow{\textit{Iso}\text{citrate dehydrogenase}} \text{Co II} \longrightarrow \alpha\text{-Ketoglutarate}$$

and

$$\alpha\text{-Ketoglutarate} + NH_3 \xrightarrow{\text{Glutamate dehydrogenase}} \text{Glutamate} + CO_2$$

so that the overall reaction is as follows :

$$\textit{Iso}\text{citrate} + NH_3 \longrightarrow \text{Glutamate} + CO_2$$

and glutamic dehydrogenase is thus seen to be concerned with the synthesis of glutamic acid.

A similar overall reaction may take place in association with ketoglutarate dehydrogenase and lipoate dehydrogenase, giving an overall reaction :

$$2\ \alpha\text{-Ketoglutarate} + \text{CoA} + NH_3 \longrightarrow \text{Succinyl-CoA} + \text{Glutamate} + CO_2$$

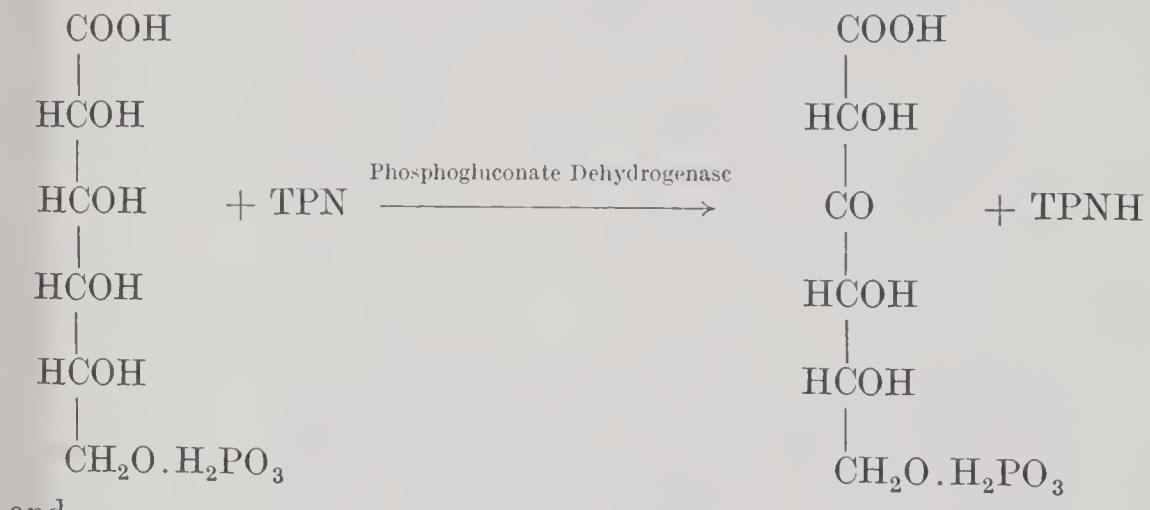
This reaction, as shown in Fig. 186, results in additional succinate for oxidations in the citric acid cycle. According to Dixon and Webb (1958) there is also a possibility that glutamate may act like Co I and Co II, that is to say as an intermediate hydrogen carrier, by undergoing alternate oxidation and reduction, catalyzed by the dehydrogenase. Finally, glutamate dehydrogenase may take part in the synthesis of urea.

The Pentose Cycle. It is now possible to demonstrate two of the enzymes contributing to the process which, in the past, has usually been referred to as the hexose monophosphate shunt. The name pentose cycle was suggested originally by Hauge, King and Cheldelin (1954) and it has now passed into common usage. The two enzymes concerned are

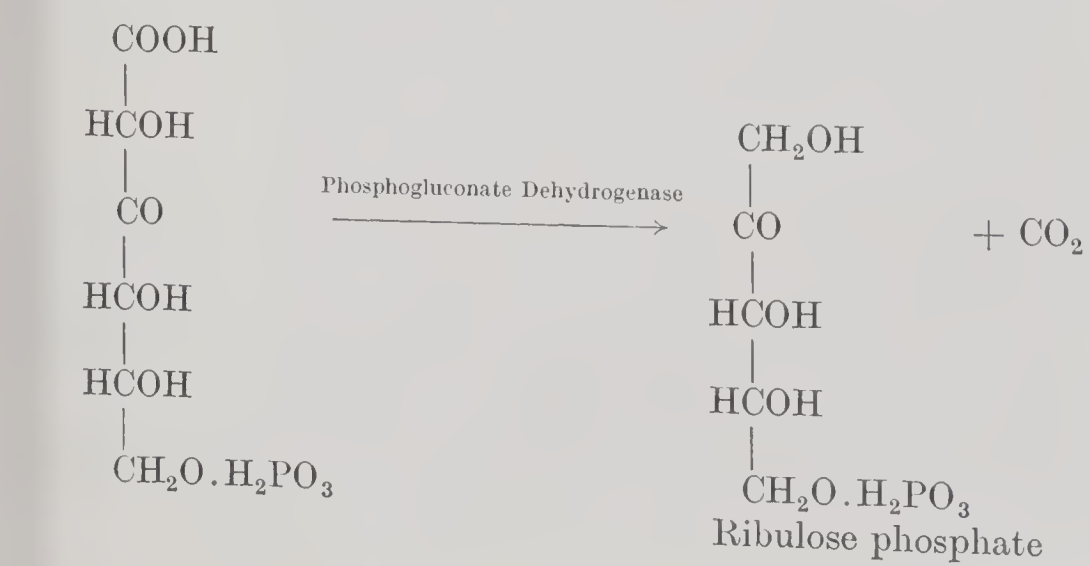
glucose-6-phosphate dehydrogenase (Phosphoglucose dehydrogenase) and 6-phosphogluconate dehydrogenase. Histochemically the association of these two is found, as expected, to be very close.

The pentose cycle has two main functions. First, it provides an alternative pathway for the oxidation of glucose, as suggested by its older name. Several groups of experimenters (Bloom, Stetten and Stetten, 1953 ; Beevers and Gibbs, 1954 ; Newburgh and Cheldelin, 1956) have discussed the contribution of the pentose cycle to carbohydrate metabolism and have made comparisons between this cycle, the Krebs' cycle and the glycolytic scheme. Scott and Cohen (1951) suggested that organisms which contain little or no Krebs' cycle activity may obtain much of their energy in this way, through the pentose cycle. The second function of the pentose cycle is that it provides a route for the synthesis of pentose nucleotides for the building up of nucleoproteins.

The first step in this latter process is the simple oxidation of phosphoglucose, by glucose-6-phosphate dehydrogenase, to the lactone of phosphogluconic acid. This compound is then split by a lactonase. The resulting phosphogluconate is oxidized and decarboxylated, probably by a single enzyme.



and



Ribulose phosphate

The product (ribulose phosphate) may be carried through the pentose cycle by a succession of activities or it may be used for the synthesis of nucleotides and thus for the production of nucleic acids.

It is relatively easy to determine histochemically whether increased pentose cycle activity is accompanied by increased nucleic acid synthesis by performing a methyl green-pyronin stain (Appendix 8, p. 825) on the serial cold microtome section, or even on the same section after removal of the formazan and simultaneous fixation by means of acetic-ethanol. Comparison with control sections usually makes the position clear. For example, in many growing tumours, and especially in squamous carcinomas, there is a greatly increased activity of glucose-6-phosphate dehydrogenase in the cells of the growing edge (Pearse, 1958 ; Scarpelli and Pearse, 1959). This is accompanied by a pronounced increase in pyroninophilia due to production of RNA. It has been suggested by Williams-Ashman (1953) that the considerable rate at which phosphoglucose and phosphogluconate are oxidized by Ehrlich ascites tumour cells indicates that the pentose cycle contributes substantially to the *respiration* of such tumours. This need not be so if the system is geared to produce RNA. Similarly, the unusually high utilization of phosphoglucose by adrenal cortical tissue has suggested to Kelly *et al.* (1955) that the pentose cycle is the most important oxidative pathway in this tissue and that it must play a significant role in adrenal metabolism. This use of the pentose cycle as the main oxidative pathway (with no increased RNA production) can be shown histochemically and it can be illustrated by another case.

When the glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity of the macula densa of the rat kidney rises sharply, as it does in hypertension caused by the application of a Goldblatt clamp (Pearse, 1958 ; Hess and Pearse, 1959), there is no corresponding rise in RNA production. One supposes that in such a case the pentose cycle provides the source of energy either for the synthesis of ATP, or for some product unknown, possibly renin. The appearance of glucose-6-phosphate dehydrogenase in the macula densa is shown in Fig. 187, p. 561, by the MTT-cobalt method.

Glycolytic Pathways. We are able to demonstrate three enzymes whose primary concern is with glycolysis. These are α-glycerophosphate dehydrogenase, alcohol dehydrogenase and lactate dehydrogenase, all three being DPN-linked enzymes. Glycerophosphate dehydrogenase takes part, with phosphoglyceraldehyde and DPN, in the metabolism of triose phosphate. Histochemically its activity can be regarded as an indication that glycerol fermentation is taking place.

Alcohol dehydrogenase is an enzyme of rather wide specificity

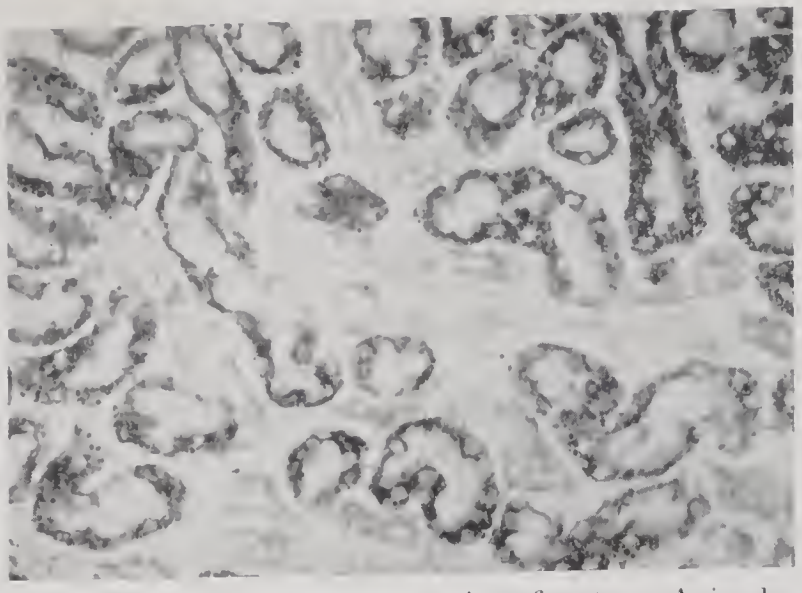


FIG. 190. Rat kidney. Juxtamedullary region of cortex. Animal maintained on Mg-deficient diet for 3 days. Swelling and increased rate of reaction can be seen in many of the mitochondria. (TPN diaphorase.) MTT-cobalt method. × 750.

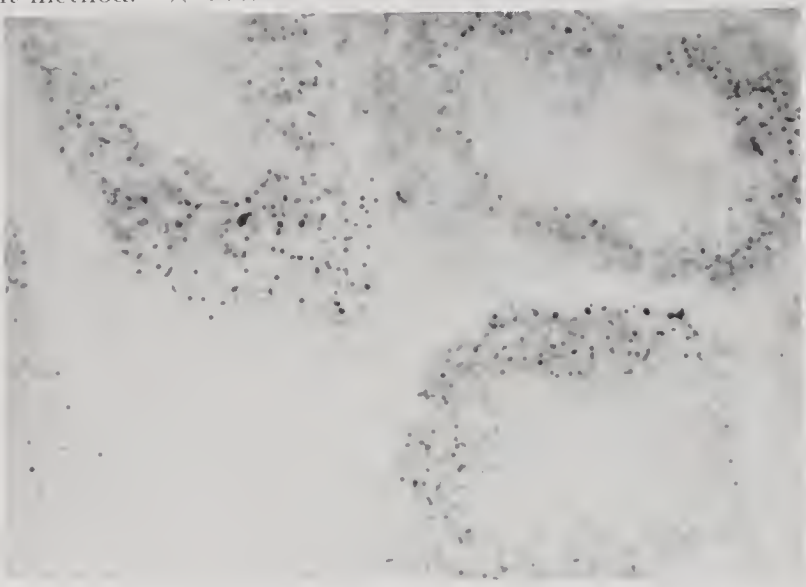


FIG. 191. Rat kidney. Succinate dehydrogenase reaction after 3 days Mg deficiency. Shows early damage to mitochondria. MTT-cobalt method. × 900.

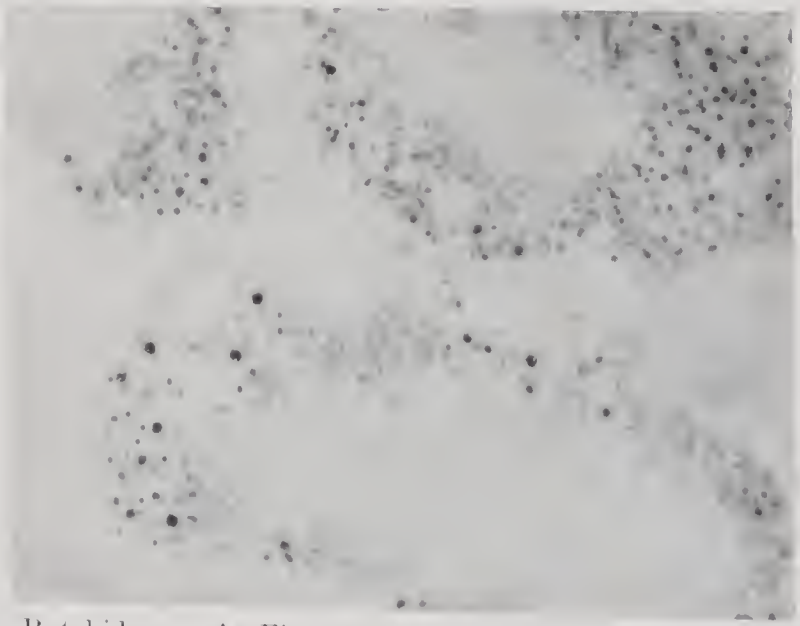


FIG. 192. Rat kidney. As Fig. 191 but section pre-incubated in hypotonic medium before succinate dehydrogenase reaction. Abnormally sensitive mitochondria show larger, darker formazan deposits. MTT-cobalt method. × 900.

[*To face p.* 576.

FIG. 193. Rat stomach. Fully protected medium for succinate dehydrogenase. Intramitochondrial localization of enzyme. MTT-cobalt method. × 4,450.

FIG. 194. As Fig. 193. Incubated in hypotonic medium for succinate dehydrogenase. Mitochondria unrecognizable. × 4,450.

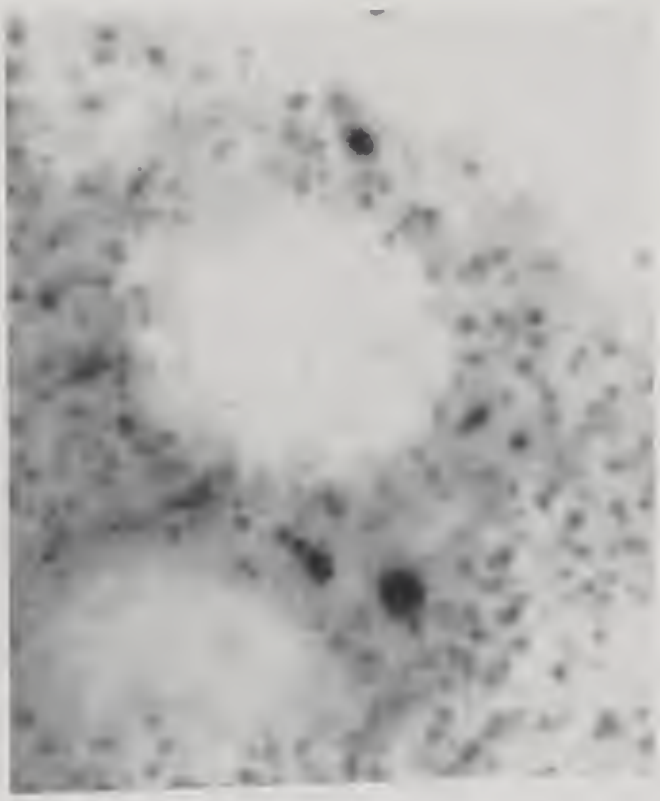


FIG. 195. Rat kidney. Distal convoluted tubule cells. Animal kept on K-deficient diet for 21 days. Shows early mitochondrial damage. MTT-cobalt method. × 4,450.

which can take part in a number of linked reactions. Primarily it is concerned either with the oxidation of alcohol or with the production of alcohol from acetaldehyde.

Lactate dehydrogenase is the most important of the three enzymes as an indicator of activity of that part of the glycolytic system in which, under anaerobic conditions in muscle, glucose is consumed and lactate produced :

$$\text{Glucose} + 2\text{ADP} + 2\text{H}_3\text{PO}_4 \longrightarrow 2 \text{ lactate} + 2\text{ATP} + 2\text{H}_2\text{O}$$

It will be observed that this reaction involves the synthesis of ATP and it thus provides the main source of energy for muscular contraction. The lactate accumulates and passes via the blood stream to the liver. Here it is oxidized, aerobically, by lactate dehydrogenase with the production of pyruvate. The latter is subsequently phosphorylated and built up into glycogen by reversal of the functions of the glycolytic scheme.

Oxidation of Fatty Acids. Fatty acids are normally oxidized completely to CO_2 and water. Under the influence of β-hydroxybutyrate dehydrogenase and DPN, β-hydroxybutric acid and acetoacetic acid are normally intraconvertible. This mechanism plays a part in the formation of ketone bodies.

Possibly the most important role of D-β-hydroxybutyrate dehydrogenase is to take part with Coenzyme A (3′-phospho-ADP-pantoyl-β-alanyl cysteamine) in the oxidation of fatty acids coupled with the synthesis of active acetate (acetyl CoA). Coenzyme A was discovered by Lipmann whose papers (1953, 1954) should be consulted for information about its activities. When D-β-hydroxybutyrate dehydrogenase acts as a D-butyrate-CoA dehydrogenase the reaction may be written :

$$\text{D-}\beta\text{-hydroxybutyryl-CoA} + \text{DPN} \longrightarrow \text{acetoacetyl-CoA} + \text{DPNH}$$

By the action of acetyl-CoA transacylase (β-ketothiolase) acetoacetyl-CoA and a molecule of CoA are converted into two molecules of acetyl-CoA. As shown in Fig. 186, this product can then enter the eitric acid cycle for oxidation.

The result of the application of the histochemical reaction for β-hydroxybutyrate dehydrogenase is shown in Fig. 188, p. 561, where the intramitochondrial localization of the formazan deposit is clearly shown. This should be compared with the similar localization of succinate dehydrogenase in a rabbit ganglion cell shown in Fig. 189. In Table 47, below, are set forth in abbreviated form the interpretations which may be attached to the various histochemieally demonstrable diaphorases and dehydrogenases.

TABLE 47

Interpretation of Diaphorase and Dehydrogenase Reactions

Substrate	Coenzyme	Mechanism or Significance
DPNH	Co I Diaphorase	Total activity of Co I-linked systems. DPN Cytochrome c reductase. Lipoate dehydrogenase.
TPNH	Co II Diaphorase	Total activity of Co II-linked systems. TPN Cytochrome c reductase.
Succinate	None	Citric Acid Cycle.
Malate	Co I	Citric Acid Cycle.
*Iso*citrate	CoI or Co II	Citric Acid Cycle. Ketoglutarate production.
Glutamate	Co I	Synthesis of amino-acids, incl. glutamate, from keto-acids + NH_3. Breakdown of glutamate. Supply of succinate for cycle. Synthesis of urea. Intermediate hydrogen carrier.
Glucose-6-phosphate 6-Phosphogluconate	Co II Co II	Pentose cycle activity. (Metabolism via Hexose Monophosphate shunt.) Synthesis of ATP. Synthesis of Nucleotides (RNA).
α-Glycerophosphate	Co I	Glycolysis (Early). Glycerol fermentation.
Alcohol	Co I	Glycolysis. Alcohol fermentation. Conversion of Acetaldehyde to Alcohol.
Lactate	Co I	Glycolysis (late) synthesis of ATP and lactate (anaerobic). Oxidation of lactate (aerobic).
D-β-hydroxybutyrate	Co I	Fatty acid oxidation ——————→ active acetate synthesis. (Acetyl-CoA.)

Some Details of Individual Dehydrogenase Reactions

As mentioned previously, the reactions for each of the above dehydrogenases result in the intramitochondrial deposition of formazan as small dots with an average diameter of 0·2 to 0·3μ. This pattern has been described for both of the diaphorases (Scarpelli, Hess and Pearse, 1958) and for succinate dehydrogenase (Pearse and Scarpelli, 1958 ; Scarpelli and Pearse, 1958). The relative activities of the various dehydrogenase systems in rat tissues have been described by Hess, Scarpelli and Pearse (1958) and it has been shown that each system has a characteristic pattern which represents only a part of

the complete diaphorase pattern given by either DPN or TPN diaphorase. Methods for all the dehydrogenases listed in Table 46 are given in Appendix 20.

Succinate Dehydrogenase (Plate VIIc, p. 545, and Fig. 189)

This is the only histochemically demonstrable dehydrogenase system which requires no coenzyme. The product (fumarate) does not inhibit the reaction and no trapping agent need therefore be employed. The rate of the reaction is increased by mid-chain block with amytal and likewise, initially, by phenazine methosulphate. Neither of these substances need be added for the routine performance of the succinate dehydrogenase reaction.

When BT was employed as electron acceptor by Rutenburg, Wolman and Seligman (1953) these authors found that certain metal ions, notably calcium, magnesium and aluminium, greatly increased the reaction rate. A similar effect was produced with bicarbonate ions and all four were therefore incorporated in the incubating medium. Using Nitro-BT, however, Nachlas *et al.* (1957) found very little improvement in staining when metal ions and bicarbonate were employed and suggested that for routine use they were unnecessary. When MTT is used these additional ions may similarly be omitted unless the mitochondria of the tissues being studied are weak in succinate dehydrogenase. In such a case aluminium ions may increase the reaction considerably.

Isocitrate Dehydrogenase

The DPN-linked enzyme has only recently been described by Plaut and Sung (1954). Histochemical activity of this enzyme is widespread though never quite so strong at its maximum as the TPN-linked enzyme. The latter, though it is only partly recoverable from the mitochondrial fraction of homogenates, is nevertheless recorded histochemically as intramitochondrial.

Malic Dehydrogenase

The DPN-linked enzyme shows strong activity in many tissues, especially smooth muscle, kidney, and salivary gland ducts. If TPN is substituted for DPN a similar but weaker pattern of activity is observed.

α-Glycerophosphate Dehydrogenase

The high activity of this enzyme in striated muscle, from a number of species in addition to the rat, has been confirmed histochemically. No oxidation occurs if β-glycerophosphate is employed. According to Ringler and Singer (1958) the α-glycerophosphate dehydrogenase

of brain mitochondria, as opposed to those of other organs, is linked to the respiratory chain without the mediation of readily dissociable coenzymes. They consider that in this instance succinate dehydrogenase and α-glycerophosphate dehydrogenase may be structurally and functionally integrated. Histochemically α-glycerophosphate can not replace succinate as electron donor in the case of rat brain mitochondria.

Glutamate Dehydrogenase

The activity of this DPN-linked enzyme was inhibited by the addition of glutamine. Replacement of DPN with TPN reduced the activity of the enzyme almost to nothing.

β-Hydroxybutyrate Dehydrogenase

No reaction was obtained with either α-hydroxybutyrate or L(+)-β-hydroxybutyrate as substrates. The histochemical reaction leading to the reduction of tetrazolium was found to be dependent solely on the oxidation of D(−)-β-hydroxybutyrate to acetoacetate. This reaction was not inhibited by dicoumarol since only the further oxidation of acetoacetate is dependent on phosphorylation, as shown by Beechey (1957). Since there is no competition between the two isomers (Lehninger and Greville, 1953) the racemic mixture of β-hydroxybutyrates can be used in place of the D-isomer.

Glucose-6-Phosphate Dehydrogenase

The localization of this enzyme in the mitochondria is at variance with information derived from homogenization studies. No explanation for this difference is forthcoming and possibly this enzyme may provide a test case for the accuracy of histochemical versus biochemical localizations.

The presence of phosphate in the incubating medium is inhibitory to this enzyme. Prolonged freezing of the tissues, as occurs with storage, was also found to diminish the activity of this enzyme more rapidly than most other systems. Inhibition is produced by ATP (0·005 M) and this effect may possibly be due to interference with oxidative decarboxylation of the product (6-phosphogluconate) which is normally carried out by the associated 6-phosphogluconate dehydrogenase. This has been shown by Glock and McLean (1953) to be totally inhibited by ATP.

Lactate Dehydrogenase

This enzyme was observed by Nachlas, Walker and Seligman (1958a) to be strongest in smooth muscle, in the gastric parietal cells and in the thick, descending limbs of Henle's loops in the kidney

(rat). The results obtained by Hess, Scarpelli and Pearse (1958) are entirely in agreement with their findings. We agree also that the overall distribution of DPN diaphorase is similar to that of lactate dehydrogenase though the relative intensities are dissimilar. This indicates only that lactate dehydrogenase is very widely distributed in a high proportion of the mitochondria of most tissues and not that it is impossible to demonstrate its precise localization.

A very interesting finding has been described by Novikoff and Masek (1958). These authors consider that in fresh frozen sections the product of incubation with Nitro-BT is not an intramitochondrial formazan deposit but a crystalline artifact lying at another level, altogether outside the mitochondria. When cold formalin-fixed sections are substituted, however, Novikoff and Masek find 1) that sufficient lactate dehydrogenase or DPN diaphorase activity survives and 2), that with Nitro-BT the resulting formazan is intramitochondrial. The production of crystalline artifacts with Nitro-BT has already been described (Chapter XIX, p. 545) and it can be entirely avoided by the use of protective media and by mounting the sections in glycerine jelly instead of dehydrating them. There is thus no need to use formalin fixation and I consider that its use prior to the demonstration of any dehydrogenase system, even if it is tough enough to survive such a procedure, is a retrograde step in dehydrogenase histochemistry which must be utterly condemned.

The production of fresh frozen sections is now a matter of such ease that fixation no longer confers even the convenience of easier handling (rather the reverse). The arguments set forth in Chapter XIX, and in this chapter, should be sufficient to make my views on this subject clear.

Alcohol Dehydrogenase

This enzyme is quite easily demonstrated histochemically but in many tissues its activity is relatively weak. Parietal cells and salivary duct cells (rat) form an exception. The most interesting application of the alcohol dehydrogenase reaction is in the retina where, as Wald (1956) has shown, the following reaction takes place.

$$\text{Vitamin A} \underset{\text{DPNH}}{\overset{\text{alcohol dehydrogenase}}{\overset{\text{DPN}+}{\rightleftharpoons}}} \text{Retinene} \xrightarrow{\text{opsin}} \text{Rhodopsin}$$

Intense alcohol dehydrogenase activity can be demonstrated in the retina of rats kept in the dark for short periods (18–24 hours). If vitamin A is used as substrate the reaction runs to the right if

hydroxylamine (or opsin) is present to trap the product. If, as in histochemical usage, alcohol is employed as substrate cyanide forms an efficient trapping agent.

Diaphorase and Dehydrogenase Reactions as Indicators of the Physical State of the Mitochondria (Mitochondrial Assay)

The appearances of the mitochondria in fresh frozen sections or smears, provided that suitable steps have been taken to preserve their integrity (Chapter XIII, p. 367) are shown in Figs. 187 and 188. On the other hand, Fig. 189 shows the effect of over-incubation in an unprotected medium. A mixture of " normal " mitochondria and swollen mitochondria containing increased amounts of formazan can be seen. The " normal " mitochondria are still intact, as can readily be ascertained by inspection with the phase microscope. If for any reason a mitochondrion is damaged before incubation, so that the membrane is abnormally permeable, the first result is that the reaction rate increases and more formazan per unit time is deposited. This produces the kind of result shown in Fig. 189 though here the cause was lack of protection and over-incubation. If, therefore, an osmotic load is put upon the mitochondria, by pre-incubating the fresh frozen sections in hypotonic or otherwise harmful media, those which are abnormally sensitive are observed to respond sooner and more violently than the normal ones.

We thus have here a tool which is capable of showing very early changes in the physiological state of the mitochondria and we can therefore indicate pathological changes in a variety of conditions a long time before any change can be seen by conventional methods. Similar anticipation is possible with the electron microscope but this hardly lends itself to routine application and selection of the sample must introduce a considerable error. With the present histochemical methods, if one mitochondrion in a section of an organ is abnormal this can be detected.

In Fig. 190 is shown the TPNH diaphorase reaction in the juxta-medullary tubules of the renal cortex of a rat maintained for 36 hours on a diet deficient in magnesium. The incubation medium was fully protective and control sections showed entirely normal mitochondria. In the magnesium-deficient kidney swollen (damaged) mitochondria are visible though sometimes there may be only one per cell. The change is more easily seen at higher magnification as in Fig. 191 (incubated for succinate dehydrogenase), and more easily still in Fig. 192 which shows the succinate dehydrogenase reaction in the same tissue pre-incubated for 30 minutes in hypotonic saline. Such treatment produces no response from the normal kidney mitochondria though it may from the more sensitive mitochondria of other

organs such as the parietal cell of the rat stomach shown in Figs. 193 and 194.

It is thus possible to calibrate every type of mitochondrion in every tissue and to apply to it just that load which will reveal the earliest change in sensitivity of the membrane. The implications of such a method in experimental pathology should be sufficiently obvious.

For all studies on the physical state of mitochondria, which are included in the comprehensive term "mitochondrial assay," the DPNH and TPNH diaphorase reactions and the succinate dehydrogenase reaction should be sufficient to cover the majority of cases. Using these reactions early changes in mitochondria have been detected, as recorded above, in magnesium deficiency (Hess *et al.*, 1959), in potassium deficiency (Fig. 195), starvation, dehydration and after poisoning with diphtheria toxin. In all cases the results have anticipated those of conventional staining methods by long periods and the sensitivity of some mitochondria to apparently trivial alterations in their environment is a truly remarkable feature, which is emphasized by the use of the techniques of mitochondrial assay.

Odd Dehydrogenases

Steroid-3β-ol Dehydrogenase

This enzyme, which is also known as β-hydroxysteroid dehydrogenase, catalyzes reactions of the type shown below :

$$\text{Testosterone} + \text{DPN}^+ \rightleftharpoons \Delta^5 \text{ androstene-3, 17-dione} + \text{DPNH} + \text{H}^+$$

Using deutero-steroid as substrate Talalay, Loewus and Vennesland (1955) showed that this reaction involves direct transfer of hydrogen from steroid to DPN. Other 3-β or 17-β hydroxysteroids are also oxidized by the enzyme which has been demonstrated histochemically by Wattenberg (1958) using 0·001 M-dehydro*epi*androsterone as substrate. According to Talalay and Marcus (1956) the substrate constant (Ks) for this compound for DPN is $1{\cdot}1 \times 10^{-5}$ M whereas that for testosterone is $5{\cdot}5 \times 10^{-5}$ M. For etiocholan-3β-ol-17-one the figure is $0{\cdot}26 \times 10^{-5}$ M which suggests that this compound should be a more efficient substrate in histochemical practice.

It is suggested by Hurlock and Talalay (1958) that the steroid dehydrogenases may function as pyridine nucleotide transhydrogenases and they consider the steroids as hydrogen carriers, or coenzymes, capable of undergoing alternate oxidation and reduction by transfer of H between DPN and TPN. Factors which favour this are the low substrate constants for both oxidized and reduced forms

of the steroid and the ready reversibility of the reaction under suitable pH conditions. Unfortunately, the pH optimum of the enzyme is 8·0 to 8·4. At pH 7.0 the forward and backward velocities of the reaction are equal and at this pH one would not expect to obtain a histochemical result unless, by trapping the product, the reaction could be made to move to the right.

It is necessary, under histochemical conditions, to run the reaction at pH 8·0 or higher to obtain reasonable reduction of tetrazolium salt. At this pH level activity of "nothing dehydrogenase" is almost maximal and I have not been able to separate this activity from steroid dehydrogenase activity using either Nitro-BT or MTT as electron acceptor. With DPN at a concentration of 0·005 M negative control sections cannot be obtained. Wattenberg (1958) used 0·001 M-DPN and obtained negative control sections. In my hands this level of coenzyme produces similarly weak results in both control and substrate-incubated tissues.

Further work on β-hydroxysteroid dehydrogenase is necessary before it can be accepted without reservation as a histochemically demonstrable enzyme.

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CHAPTER XXI

PEPTIDASES AND OTHER ENZYMES

This chapter is mainly concerned with an important group of enzymes, the peptidases, which hydrolyse —CO—NH— linkages. Histochemical representation within this group is at the present time restricted to the direct demonstration of a single enzyme, leucine aminopeptidase, and the indirect demonstration of cathepsin C activity by means of its esterolytic actions. There is little doubt, however, that new peptidase substrates will be developed in the near future and that we shall be able to cover the field much more effectively in a few years time.

A second group of hydrolytic enzymes, the sulphatases, are also dealt with in this chapter since they do not fit readily into any of the preceding chapters on enzyme histochemistry. Our studies in this field are largely restricted to the single group of aryl sulphatases although a method for myrosulphatase exists which at least has the merit of a respectable antiquity.

Finally, we have to consider the position of histochemical methods designed to localize carbonic anhydrase. These likewise do not fit into any of the preceding chapters, but they are not, at present, of sufficient importance to warrant a separate chapter.

Properties of Leucine Aminopeptidase

This enzyme, which is also known as cathepsin III, is present in the intestinal secretions of most animals and also, intracellularly, in a number of their tissues. It hydrolyses a variety of peptides having a free α-amino group on a terminal leucine or other related amino-acid. Its occurrence in the tissues, and some of its properties, have been described by Berger and Johnson (1940) and also by Johnson and Berger (1942), Fruton (1946), and Smith (1948, 1951). The properties of the enzyme, isolated from pig kidney by Spackman, Smith and Brown (1955), have been described by Smith and Spackman (1955). According to these authors it has a broad specificity for leucyl and other compounds, is activated by Mn^{2+} and Mg^{2+} ions and inactivated by versene and citrate. These properties had previously been recorded by Berger and Johnson (1939) and by Smith (1948).

Extracts of pig intestine split leucinamide, leucyl glycine and leucylglycyl glycine at almost equal rates but during the process of purification (Smith and Bergmann, 1944) different activities towards these three compounds appear. It had been shown by Smith (1948)

that a preparation from human uterus could split leucyl glycine four times faster than leucylglycylglycine and it was suggested, therefore, that this tripeptide was hydrolysed by another enzyme as well as by leucine aminopeptidase. As far as we are concerned, the important point (Smith, 1951) is that only leucine aminopeptidase is capable of splitting leucinamide and, by inference, only this enzyme should hydrolyse L-leucyl-β-naphthylamide.

Methods for Leucine Aminopeptidase (DW. 38)

Development of Methods for Aminopeptidases

The first histochemical substrates for peptidase activity were produced by Gomori (1954a and b). Following the observation by Greenstein and his associates (Birnbaum *et al.*, 1952 ; Rao *et al.*, 1952) that chloroacetyl amino-acids were hydrolysed more rapidly than the corresponding acetyl or glyceryl derivatives Gomori (1954a) tested the chloroacetylnaphthylamines as histochemical substrates. He found that these were very rapidly hydrolysed by cold acetone-fixed tissues and that they could form the basis for a histochemical method, using Garnet GBC as trapping agent for the free naphthylamines. Later (1954b) Gomori prepared glycyl-α and β-naphthylamines, and α and β-alanyl-β-naphthylamines, as chromogenic substrates for aminopeptidases. Once again he used cold acetone-fixed tissues and Garnet GBC and observed that the localization of activity was similar to that obtained with the chloroacetyl naphthylamines.

A new chromogenic substrate for aminopeptidases was synthesized, independently, by Green *et al.* (1955) and by Folk and Burstone (1955). Directly continuing the studies initiated by Gomori, and recorded above, Burstone and Folk (1956) made histochemical comparison of various substrates. They found that considerable improvement in the histochemical localization of aminopeptidases could be obtained with either L-leucyl-β-naphthylamide or DL-alanyl-β-naphthylamide. Marked inhibition was observed with Cu^{2+}, Pb^{2+} and Ca^{2+} ions, which are known inhibitors of leucine aminopeptidase, and activation was produced by the addition of cyanide (0·003M) to the incubating medium. No activation occurred, however, with Mg^{2+} or Mn^{2+}. The authors presumed that this last finding was due to the fact that in freeze-dried sections, which they preferred and recommended, these metal ions were already present in optimal concentration.

The results of the application of Burstone's technique are shown in Plate VIIIA, p. 656. The red dye produced in freeze-dried tissues by coupling with Garnet GBC is orange coloured in fresh frozen sections and, in the case of the latter, large crystals are rapidly formed. In freeze-dried sections the precipitate is much smaller and may remain

stable for months. According to Burstone, diazonium salts containing zinc as stabilizer cannot be used in his method since zinc ions are powerful inhibitors of aminopeptidase.

Methods "Specific" for Leucine Aminopeptidase

A method basically very similar to Burstone's was produced by Nachlas, Crawford and Seligman (1957) who also employed L-leucyl-β-naphthylamide as substrate. Since the main specificity requirements of the enzyme are provided by the leucine residue and the group linked at the leucine carboxyl is known to exert only a minor influence these authors considered that the substrate they used would be hydrolysed by leucine aminopeptidase (LAP). The fact that similar, though not identical, tissue localizations can be obtained when non-leucyl naphthylamides are employed as substrates need not produce any lack of confidence in this view since other aminopeptidases may well have identical localizations. It is certainly convenient, even if not absolutely proven, to regard the hydrolysis of L-leucyl-β-naphthylamide in tissue sections as due to LAP activity.

pH Optimum. Nachlas and his colleagues noted that the histochemical reaction for LAP could not be carried out at the pH optimum of the enzyme (pH 8–9·5) which has a broad range of activity from pH 6·5 to 9·5. Folk and Burstone (1955) found an optimum pH activity, under histochemical conditions, at pH 7·0 to 7·4, while Green *et al.* (1955) observed an optimum at pH 6·8. Using the same substrate *in vitro* the optimum pH was 8·0.

An explanation for these findings lies in the inhibitory activity of the diazonium salts and possibly their accompanying metal ions. Nachlas, Crawford and Seligman (1957) noted that Fast blue B (6)* produced 60 per cent. inhibition at pH 6·5 and 100 per cent. at pH 8·0, while Fast Garnet GBC (18)* caused only 30 per cent. inhibition at both pH levels.

Choice of Diazonium Salt. In spite of the apparent advantage of GBC over Blue B salt the former has three severe disadvantages which make its use, except perhaps with freeze-dried sections, impossible. The first of these is that although *in vitro* the coupling rate appears to be faster than that of Fast blue B, in tissue sections it appears to be slower. Diffusion of the free β-naphthylamine is apparently greater. The second disadvantage of Garnet GBC is that the azo dye which it produces gives large crystals in a very short time except, as mentioned above, in freeze-dried sections. The third disadvantage is that metal chelation of the azo dye from Garnet GBC cannot be obtained.

Metal Chelation. The long molecule produced from β-naphthylamine with the dicoupler Fast blue B is moderately substantive for protein

* These numbers refer to the Table of Diazonium Salts in Appendix 14.

and crystallization is thus prevented. When Cu^{2+} ions are added, after incubation, the azo dye from β-naphthylamine and blue B is able to chelate 2 atoms of the metal per molecule of dye. This results in the formation of a stable copper chelate which is deep bluish-purple in colour compared with the red of the unchelated dye.

Other diazonium salts containing a methoxy group *ortho* to the diazonium were also tested (Fast red B and Fast black K salts). These, being mono-couplers, produced azo dyes which chelated only one atom of copper per molecule and which were far less substantive. No diazonium salt was found which coupled rapidly enough to prevent diffusion entirely. Details of this method are given in Appendix 21, p. 913, and the result is illustrated in Fig. 196, p. 592.

A Modified Method for Leucine Aminopeptidase

A new substrate for the histochemical demonstration of LAP has been synthesized by Rosenblatt, Nachlas and Seligman (1958). This substrate, L-leucyl-4-methoxy-β-naphthylamide, was designed to couple more rapidly with the diazonium salt and thus to prevent the diffusion artifacts from which both of the older methods suffer. A preliminary report of the histochemical use of this substrate was given by Nachlas, Rosenblatt and Seligman (1958) and a fuller description has now been given by Nachlas, Rosenblatt, Monis and Seligman (1958).

The diazonium salt of choice remains Fast blue B and copper chelation results in a stable pigment which is no longer lipid soluble. The red colour of the metal-free dye is in this case, however, substantially unaltered. The result is illustrated, in the case of the rat parathyroid gland, in Fig. 197, p. 592. This should be compared with Fig. 196, which illustrates the use of the older method on the same tissue. The degree of improvement is obviously very considerable.

This new method should provide a strong stimulus for the histochemical exploration of changes in LAP activity in various physiological and pathological conditions. A small amount of work has already been done, mainly with one or other of the older methods. Burstone (1956) described the distribution of proteolytic enzymes in human neoplasms with particular reference to the high stromal activity in the vicinity of tumours. His evidence was not entirely in agreement with that produced by Sylvén and Malmgren (1955), who used biochemical methods. Braun-Falco (1957) also used the LAP method and suggested that aminopeptidase activity might be connected with the invasiveness of tumours. This author (Braun-Falco and Salfeld, 1959) has drawn attention to the well-known fact that the mast cells of many species contain a strong aminopeptidase activity, and has suggested that this is connected with release of heparin from its lipoprotein complex. It is of interest that Benditt (1956), using

chloracetyl-2-hydroxy-3-naphthoic acid as substrate, found a strong enzyme activity in mast cells which he suggested resembled that of chymotrypsin. The mast cells offer an object for studies which could not be undertaken by biochemical methods. These are (usually) incapable of distinguishing the activity of one cell from another. The activity of LAP in the production of parathyroid hormone has been considered by Pearse and Tremblay (1958) who have demonstrated a presumptive direct relationship between the two. In the inactive parathyroid gland of rats treated with dihydrotachysterol LAP activity fell to less than half the resting value for the normal gland (estimated biochemically). In rats maintained on a low calcium diet there was a marked increase in LAP activity. It was therefore assumed that the enzyme is closely connected with the production of parathyroid hormone and that the peptide hormone, or its carrier protein, might well be found to possess a terminal leucyl group. Recently Willighagen and Planteydt (1959), using Burstone's method, have observed a strong LAP in human gastric and bile duct tumours which is absent from other types of tumour. According to these authors normal human stomach contains no LAP. In the rat, however, strong activity is present in the basal portion of the gastric glands (Nachlas, Crawford and Seligman, 1957). Willighagen and Planteydt make the suggestion that examination of lymph nodes, or other tissues, by the LAP method may enable one to pick out malignant cells of gastric origin. Their suggestion is an interesting one but needs to be qualified by a full tumour survey.

A Possible Method for Cathepsin C (DW. 11)

A number of proteolytic enzymes are capable of splitting esters as well as peptides (Neurath and Schwert, 1950). Peptidases like chymotrypsin and pepsin (but not trypsin) show a marked preference for links involving aromatic amino-acid residues and for both the best substrates are esters, especially those of N-acyl-tyrosine. Cathepsin C is one of the intracellular proteolytic enzymes which act on proteins or synthetic amino-acid esters at acid pH levels. A new classification of the animal cathepsins has been proposed by Tallan, Jones and Fruton (1952) which is based on their substrate specificities and cathepsin C is found to be homospecific with chymotrypsin.

An organophosphorus-resistant indoxyl esterase has been described in rat kidney and other tissues by Hess and Pearse (1958). Its properties closely resemble those of cathepsin C. This enzyme was found in macrophages, Kupffer cells (Fig. 198), islets of Langerhans, and in the epithelium of the pancreatic ducts, as well as in the kidney. It is probably identical with the non-specific esterase described in pituitary mucoid cells by Pearse (1956) and in rat brain and rat thyroid (Pepler

and Pearse, 1957a and b) as well as in human argentaffinomas (carcinoids) by Pearse and Pepler (1957).

Myers *et al.* (1955) suggested that the E600-resistant and heat-resistant esterase of rat pancreas might be related to a proteolytic enzyme, different from trypsin or chymotrypsin. The fact that the latter is able to hydrolyse *o*-acetylindoxyl supports the assumption that cathepsin-like activity may be responsible for part of the histochemically observed hydrolysis of indoxyl esters. The rat kidney enzyme was observed by Hess and Pearse (1958) to be heat-resistant, optimally active at pH 5·3 and to be activated by ascorbic acid, cysteine and glutathione. These are known to have a similar effect on cathepsins (Smith, 1951). The inhibition observed with iodoacetate, D-amino-acids, and cyanide is a property of the carboxypeptidases (Elkins-Kaufmann and Neurath (1948). Since the latter are metalloenzymes they are inhibited by citrate and a similar inhibition was noted under histochemical conditions.

Phenylmercuric ions activated the enzyme under histochemical conditions as well as *in vitro* and this suggests a similarity with the "C-esterase" described by Bergmann, Segal and Rimon (1957). Strong inhibition by β-phenylpropionic acid suggests a chymotrypsin-like activity. Strong activation by detergents (Tweens 60 and 80) resembles the effect of these compounds on carboxypeptidases and a similar activation by pretreatment with trypsin at pH 7·8 suggests that part of the enzyme is present as an inactive precursor.

Although the case for regarding the heat-resistant organophosphorus-resistant indoxyl esterase of mammalian tumours as a cathepsin cannot be considered to be conclusively proven, the available evidence suggests that cathepsin-like activity can be demonstrated histochemically by means of esterase substrates. The best of these, at least for work on cathepsins, are undoubtedly the halogen-substituted indoxyl esterases. Now that these are commercially available their use is no longer prevented by synthetic difficulties. The method, as applied to cathepsins, is described in Appendix 21.

Development of New Techniques

The possibility of using the synthetic, rather than the hydrolytic effect of peptidases, in developing a histochemical method for their demonstration seems to have been overlooked. The description by Murachi (1956a and b) of the enzymic synthesis of acyl lysine anilides suggests a possible basis for a histochemical method.

$$\text{Acylamino acid} + \text{aniline} \overset{\text{Enzyme}}{\rightleftharpoons} \text{Anilide}$$

If the solubility of the anilide is slight the reaction moves to the right, as for instance with α-butyryl-ε-benzoyl-DL-lysine. The develop-

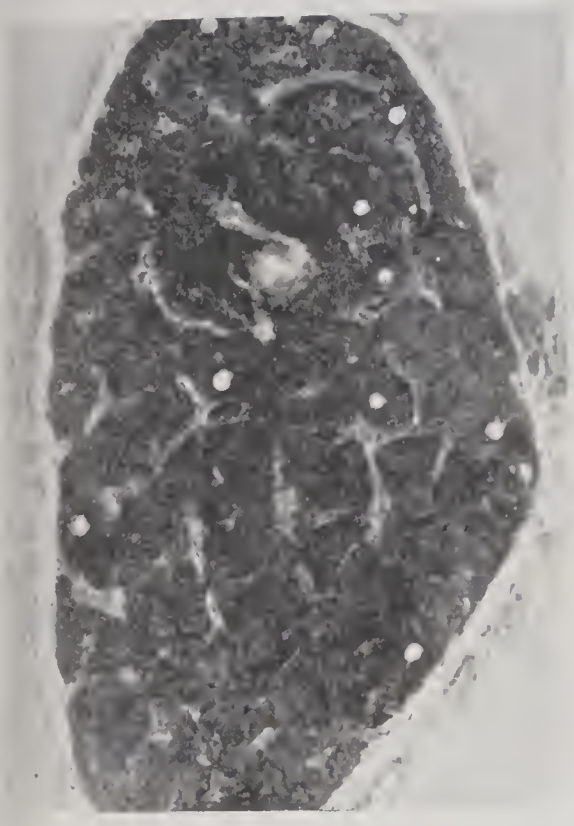


Fig. 196. Rat parathyroid gland. Embedded in liver and cut on eold microtome. Distribution of leucine aminopeptidase using L-leucyl-β-naphthylamide and Fast blue B salt. (Post-chelated with copper.) Note diffusion at edge of gland. × 120.

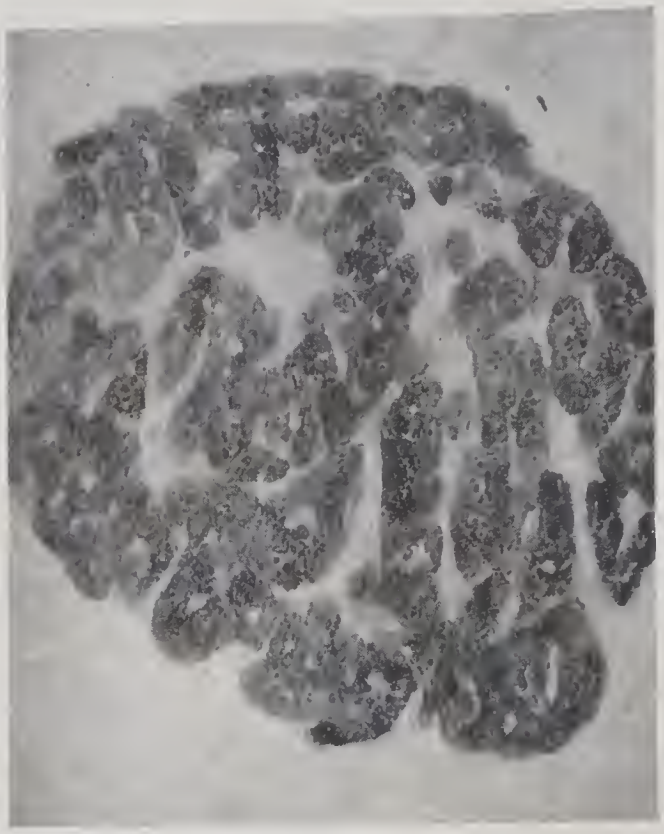


Fig. 197. As Fig. 196. Distribution of leucine aminopeptidase using L-leucyl-4-methoxy-β-naphthylamide and Fast blue B salt. (Post-ehelated with eopper.) Note absence of diffusion. × 120.

Fig. 198. Rat liver. E 600-resistant esterase in Kupffer cells which is probably a cathepsin. 4-Chloro-5-bromoindoxyl acetate. × 550.

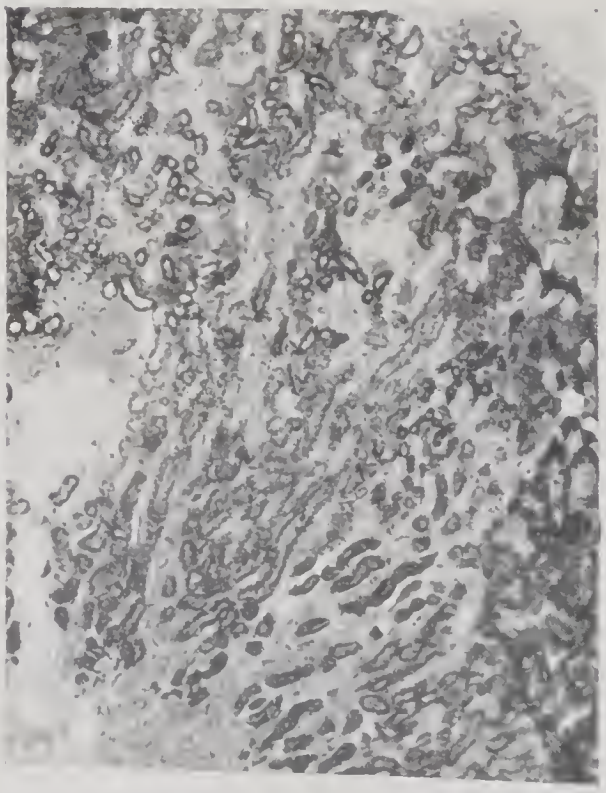


Fig. 199. Mouse kidney. Localization of arylsulphatase using Naphthol AS sulphate and Fast red TR salt. × 47.

[To face p. 592.

ment of an insoluble product would seem to provide the requisite qualities both kinetically and histochemically.

The Sulphatases

There are four known types of sulphatase in animal and plant tissues and these are known as arylsulphatase, glucosulphatase, chondrosulphatase and myrosulphatase. The gluco- and chondrosulphatases are not present in the tissues of higher animals (Fromageot, 1950), and although myrosulphatase has been found in liver, muscle, kidney and other organs of the horse and rabbit, the only histochemical attempt at its demonstration was that of Peche (1913), until two methods were evolved by Ohara and Kurata (1950). These authors trapped the inorganic sulphate with lead ions and converted the product subsequently to lead sulphide. The natural substrate of this enzyme, sinigrin,

$$\begin{array}{l} C_6H_{11}O_5SC = N.C_3H_5 \\ \quad\quad\quad\quad\quad\quad | \\ \quad\quad\quad\quad\quad\quad O\ SO_2OK \end{array}$$

obviously lends itself to a procedure of the Gomori type, precipitating the released $KHSO_4$ with barium salts, for instance. The only other sulphatase for which histochemical methods have been employed is arylsulphatase. Attempts by Dohlman and Friedenwald (1955) to demonstrate chondrosulphatase in mammalian tissues, with heparin or chondroitin sulphate as substrates, were completely unsuccessful.

This enzyme was described by Neuberg and Kurono (1923) in taka-diastase and it also occurs in *Murex*, where it is responsible for the hydrolysis of potassium indoxyl sulphate. According to Huggins and Smith (1947) arylsulphatase is found in rat tumours in far higher concentrations than in normal tissues. A transmissible rat sarcoma, for instance, contained 0·8–1·7 units of enzyme per mg. of tissue, while the connective tissue of origin contained 0·2–0·02 units per mg. only. Several substrates have been used for the chemical assay of arylsulphatases. These are potassium *p*-nitrophenylsulphate, used by Huggins and Smith, potassium indoxylsulphate used by Neuberg and Wagner (1925), nitrocatechol sulphate, and potassium *p*-acetylphenyl sulphate. The principles involved in the use of these have been the basis of two different histochemical methods.

Since the first edition of this book appeared a very considerable amount of biochemical work has been done on the arylsulphatases of mammalian tissues, chiefly by two groups of workers. The complex nature of arylsulphatase in ox liver was discussed by Roy (1953) who found that two fractions could be extracted from homogenates. One, having a pH optimum of 4·7 in acetate buffer, had an optimal substrate

concentration of 0·003 M (nitrocatechol sulphate) whereas the second was most active at pH 5·7 and showed an anomalous response to variations in substrate concentration. Dodgson, Spencer and Thomas (1953), using *p*-acetylphenyl sulphate as substrate found that for rat liver sulphatase the pH optimum was 7·2 in acetate buffer. Activity in the case of the male rat was significantly higher than in the female.

In a much later paper in this series Dodgson, Spencer and Wynn (1956) divided the arylsulphatase activity of ox, rat and human livers into three distinct enzymes, as originally suggested by Roy (1953, 1954). Two of these enzymes (the sulphatases A and B of Roy) were readily solubilized and found to be associated with the mitochondrial fraction. The third (C) was considered to be microsomal in origin.

A survey of the distribution of arylsulphatase in the tissues of seven mammals was made by Rutenburg and Seligman (1956) but in this work no allowance was made for the existence of three different varieties of the enzyme. In his earlier work Roy (1953, 1954) had indicated that his sulphatases A and B hydrolysed nitrocatechol sulphate rapidly but *p*-nitrophenyl sulphate hardly at all. His third enzyme, arylsulphatase C (Roy, 1956b) hydrolysed the latter substrate very rapidly. Sulphatases of the type of ox liver sulphatase B have been found in all vertebrate species examined (Roy, 1958) and in higher species this enzyme is accompanied by sulphatases A and C. Only in the guinea-pig does sulphatase A occur without the simultaneous occurrence of sulphatase C. Extreme variations are found in the distribution of the various types of enzyme, however. In the rat the greater part of the total sulphatase is type B whereas in the ox type A activity predominates. Species lacking in sulphatase C also lack the steroid sulphatase activity described by Roy (1956a, 1957).

Possible Functions of Arylsulphatases

There are few indications of the possible functions of the various arylsulphatases. As mentioned above, sulphatase C can act as a steroid sulphatase and Roy (1956a) has shown that this enzyme can synthesize dehydro*epi*androsterone sulphate. Like the sulphatase described by Dodgson *et al.* (1953) it is much stronger in the male than in the female.

Working with invertebrates, Suzuki, Takahashi and Egami (1957) have shown that the sulphated polysaccharide of the mucous gland of *Triton nodiferus* (*Charonia lampas*) can be sulphated by a system consisting of an arylsulphate, an arylsulphatase, a carbohydrate factor and at least one unknown factor. The physiological significance of this system is not clear, but, if similar systems exist in other animals, there should be present arylsulphates whose specific function is to act as sulphate donors. Dodgson, Rose and Tudball (1959) had considered

that tyrosine-*O*-sulphate might act in this way, but their studies showed that it was not attacked by sulphatases B and C and only slowly by sulphatase A. The search for an arylsulphate sulphate donor continues therefore.

Histochemically, as will be seen, we are much behind the biochemists in the matter of the sulphatases. Until we can make clear distinction between the three types of enzyme it is useless to expect much progress in the interpretation of results.

Methods for Arylsulphatases (DW. 137)

Methods Using Naphthyl Sulphates

Seligman *et al.* (1949) endeavoured to produce a simultaneous coupling azo dye method for the demonstration of sulphatases using α- and β-naphthyl sulphuric acid esters. The former were hydrolysed exceedingly slowly and were, therefore, histochemically unsuitable. With the latter, although hydrolysis occurred readily enough, the authors found that coupling with a diazonium salt at pH 4·5 would not occur. Since the optimum pH for arylsulphatases was shown by Huggins and Smith (1947) to be in the region of pH 6·12, and since there are several diazonium salts which will couple with naphthols at this pH, I endeavoured to produce a simultaneous coupling azo dye method, returning to the principle abandoned by Seligman *et al.* (1949). Using 0·01 M-potassium 2-naphthyl sulphate as substrate, maintained at pH 6·12 with 0·5 M sodium acetate-acetic acid buffer, and incubating cold formalin-fixed frozen sections of rat tissues for as long as 24 hours at 37°, no azo dye precipitate was obtained in any case. The diazonium salt usually employed, the diazotate of 4-benzoylamino-2 : 5-dimethoxyaniline, has been shown to be extremely stable at pH 6·12 and it will couple with β-naphthol at this pH. Temperatures of 60° for 4 hours were even employed, again without success, but without appreciable discoloration of the background structures with breakdown products of the diazotate.

Post-coupling Azo Dye Method

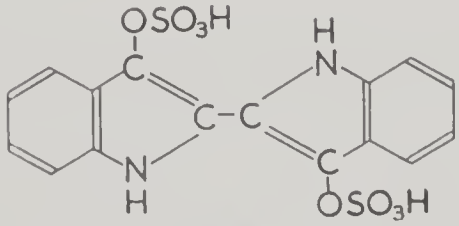
Seligman and his colleagues subsequently evolved a post-coupling method using potassium 6-bromo-2-naphthyl sulphate as substrate and incubating at pH 4·5–6·0 at 37° for a suitable period. After washing, the final coupling with diazotized *o*-dianisidine was performed at pH 9·0, to give a blue insoluble azo dye. Seligman, Nachlas and Cohen (1950) made brief mention of an alternative substrate for sulphatases (potassium 6-benzoyl-2-naphthyl sulphate) with which they used a similar post-coupling technique. The method was fully described by Rutenburg, Cohen and Seligman (1952), who used the

benzoyl compound with formalin-fixed or fresh frozen sections at pH 6·1. The incubation period was 2–3 hours with active tissues and subsequent coupling with Fast blue B produced red (monocoupling) and blue (dicoupling) products. In my hands the use of this method gives rise to red rather than blue products and since these are soluble in lipids the general localization of the enzyme is poor. The only recorded studies in applied histochemistry of the sulphatases have been made with post-coupling techniques using either 6-bromo or 6-benzoyl-2-naphthyl sulphate. Using the former, for instance, Hayashi *et al.* (1957) found cyclical changes in the surface layers of rat endometrium. Although I consider these methods unsatisfactory, nevertheless details are given in Appendix 21, p. 914.

Alternative Methods

Naphthol AS-sulphates. Using Naphthol AS-sulphate synthesized by the method of Rath and Feess (1954), Goessner (1958) was able to demonstrate the localization of arylsulphatase at pH 6·2 with Fast red TR salt (9) in a simultaneous coupling method. The results he obtained, which are illustrated in Fig. 199, p. 592, in the case of mouse kidney, are sufficiently promising to warrant further study.

Indoxyl Sulphates. Potassium indoxyl sulphate itself does not seem to have been used as a histochemical substrate for arylsulphatase. Recently, however, Sunder and Sunder (1950) employed the disulphate ester of leuco-indigo (Indigosol O) as a substrate for enzymes present in tumours.



Indigosol O.

A deposit of indigo was produced which was ascribed by the authors to the presence of oxidases. As Holt (1952) has pointed out, the result may possibly have been due to sulphatase activity, although the diacetate of leuco-indigo, for instance, is not hydrolysed by esterases.

Carbonic Anhydrase (CAH)

This enzyme, which has since been shown to be a zinc-protein, was discovered by Meldrum and Roughton (1932, 1933) in red blood cells. It can be regarded either as a hydrating enzyme or as a carbon dioxide-adding enzyme. Its occurrence and characteristics have been fully described by Roughton and Clark (1951).

By the action of the enzyme carbonic acid is split to yield CO_2 and H_2O and, in the reverse direction, CO_2 is hydrated to carbonic acid.

$$O=C=O + H{-}O{-}H \underset{CAH}{\rightleftharpoons} O=C\begin{matrix}OH\\OH\end{matrix} \underset{\text{spontaneous}}{\rightleftharpoons} O=C\begin{matrix}O^-\\OH\end{matrix} + H^+$$

Carbonic anhydrase is responsible for the capacity of the blood to carry CO_2 away from the tissues and the reaction, in this case, proceeds from left to right. In the kidneys of some animals, where it is present in the convoluted tubules, it is considered to play an important role in the maintenance of the pH of the blood, providing the bicarbonate which exchanges Na^+ for H^+.

Method for Carbonic Anhydrase (DW. 620)

The first published histochemical technique for CAH was that of Kurata (1953) who incubated cold acetone-fixed tissue slices in a bicarbonate mixture containing $MnCl_2$. After washing, the tissue was embedded in paraffin wax. Subsequently sections were cut and treated with potassium periodate in order to render visible the precipitates of manganese carbonate. Kurata suggested that calcium, cobalt, or nickel could all be employed in place of manganese.

Most workers were unable to obtain satisfactory results with the original method of Kurata but modifications using the cobalt system were employed by Goebel and Puchtler (1954) and by Braun-Falco and Rathgens (1955a). In the modification introduced by the latter authors a buffered bicarbonate solution containing cobalt ions was used and the reaction taking place in the tissues was supposedly :

$$Co(HCO_3)_2 \overset{CAH}{\rightleftharpoons} CoCO_3 + H_2O + CO_2$$

The precipitated cobalt carbonate was subsequently converted to the sulphide and thus rendered visible. The results, in my hands, were always very dirty with fine and coarse amorphous precipitates covering the entire section at times. Although the specificity of the method was supported by the observed inhibition with cyanide, azide or sulphide positive reactions could be obtained in inactivated control sections and in tissues incubated in the absence of bicarbonate. The specific CAH inhibitor Diamox (2-acetylamino-1,3,4-thiadiazol-5-sulphonamide) was used by both Puchtler and Ranniger (1955) and by Braun-Falco and Rathgens (1955b), who reported that no inhibition of the reaction occurred. A possible explanation for this last observation is the insolubility of Diamox in water. Hausler (1958), using the soluble sodium salt (Diamox parenteral-Lederle) reported the successful inhibition both of the tissue reaction and also of the activity of a purified CAH (Cartase-Schering), adsorbed on filter paper. Modifications of the cobalt method which were introduced by Hausler success-

fully eliminated the troublesome precipitates associated with the older methods. This was achieved by dissolving the bicarbonate-cobalt in sodium sulphate and adding 0·05 M H_2SO_4. The strongest reaction in rat tissues was in the pars recta of the proximal tubules.

The mechanism of the reaction was explained by Hausler as follows :

$$2\,HCO_3^- \rightleftharpoons CO_3^{2-} + H_2CO_3$$

$$H_2CO_3 \overset{CAH}{\rightleftharpoons} H_2O + CO_2$$

The CO_2, produced by the second, enzyme-catalyzed reaction, escapes and the equilibrium of the first reaction, which is spontaneous, shifts to the right. Large numbers of CO_3^{2-} ions are produced and these are precipitated by the Co^{2+} ions present in the medium. The application of Hauser's modification of the Kurata method results in the production of clean sections, but any localization which results is histological rather than cytological. A puzzling feature is the strong reaction in the protein of the tubular lumen with a negative reaction in the cells themselves.

The question of the specificity of the histochemical reaction has been taken up by a number of workers. After testing sections from kidney and pancreas in seven mammalian species Fand, Levine and Erwin (1958, 1959) suggested that the final localization of CoS might be connected with the presence of zinc. This hypothesis arose from the observation that in the pancreas staining by the modified Kurata method occurred in the islets whereas, according to biochemical evidence, pancreatic CAH is situated in the acini.

Using a Coujard slide technique Fand and her associates found that zinc, manganese, cadmium and other divalent metal ions gave strongly positive staining. A positive correlation with dithizone staining for zinc (Chapter XXIV, p. 707) was obtained in the islets but kidney tissue was not stained by this reaction. It was concluded that the Kurata method did not demonstrate CAH.

In spite of the foregoing strictures I include the Hausler modification of the Kurata method in Appendix 21 since many workers will no doubt wish to test the reaction for themselves. My own view is that, although the explanation offered by Fand cannot explain all the known facts about the histochemical reaction, it is necessary to agree with her conclusion that the Kurata reaction does not indicate CAH.

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CHAPTER XXII

ENZYMES AS HISTOCHEMICAL REAGENTS

Introduction

It will be appreciated, from what has already been stated in Chapter I, that the use of enzymes for what was originally called "enzymal analysis" is nearly as old as the science of histochemistry. Many of the modern techniques in this field are essentially those used by the older experimenters except that their crude extracts have been replaced by more or less purified crystalline enzymes. The use of these represents a very great technical advance, but a crystalline enzyme is not necessarily pure, nor is its activity necessarily specific. Danielli (1946, 1947) rightly stressed these points and maintained that there was no known method for establishing the specificity of any enzyme for a single substrate or group of substrates. A third point to which attention was drawn by this author was the importance of accessibility of the substrate in the customary tissue preparations. He claimed that a monomolecular layer of protein covering a nucleic acid would prevent access of the specific nuclease, for instance. It is clear that this particular objection is not of much importance in practice, since the results of enzyme action on intact cells in smears, and on similar cells in tissue sections, do not differ appreciably.

In addition to its purity, a large number of factors are capable of modifying the results obtained when an enzyme is allowed to react on a tissue preparation. Some of these affect the enzyme itself and others the substrate. Kaufmann, Gay and McDonald (1950) stress the fact that the use of enzymes for accurate histochemical analysis demands control of all the variables capable of modifying the reaction, those affecting the process of hydrolysis and those affecting subsequent staining alike. The most important factors in the first class are the concentration of the enzyme, the time and temperature of incubation, the pH at which it is carried out, the presence of electrolytes, activators and inhibitors, and the stability of the enzyme under these conditions. In the second class the most important considerations are the fixative used to preserve the tissues and the stain finally employed to demonstrate the removal of substrate by the enzyme.

Until quite recently it was usual for enzymes to be dissolved in aqueous buffer solutions at their optimum pH and at a concentration more or less empirically determined. The investigations of Stowell and Zorzoli (1947), which are more fully referred to below, showed

that the presence of electrolytes could exert a marked influence on the amount of material removed by enzyme action (in this case ribonuclease). Effects of this kind can be avoided by employing solutions of enzyme in glass-distilled water. The pH of these is in the region of neutrality, though often slightly on the acid side. The temperature of incubation is now usually maintained at 37°, which is optimal for most of the enzymes with which we are concerned, though in some cases lower temperatures are quite adequate and in one case the optimum is much higher. The time of incubation is varied more or less empirically but mere lengthening of the incubation period does not suffice to produce the desired result if inactivation of the enzyme is rapid. Such is the case in some instances referred to below.

Inhibitors, often essential in histochemical localization of enzymes, have not been used in analytical procedures, although the inactivation of undesirable members of enzyme mixtures might be obtained in this way. The presence of natural inhibitors in the tissue sections, however, may modify the reaction considerably. In this category mercury and chromium (derived from the fixative) are important. Activators other than inorganic ions are seldom employed in analysis with enzymes and a low concentration of the necessary ion can usually be supplied without affecting the action of the incubating medium on the substrate other than by true activation of the enzyme.

The effect of the fixative employed is in all cases of paramount importance. In the first place it is necessary that the concentration and localization of the substrate shall be as perfectly preserved as possible, and morphological considerations have therefore influenced the choice of fixative more than they would have done if a strictly histochemical view had been maintained. Secondly, it is necessary that no blocking effect should prevent access of the enzyme to its substrate in the tissues and that, granted full access, no inhibitory effect should modify its action. Thirdly, the substrate should be so preserved that the reaction product is entirely free to diffuse from the tissues. At first sight it would appear that freeze-drying offers the best chance of satisfying these criteria, but in many cases the use of unfixed material is entirely prohibited by the fact that the substrate present in the tissues is partially or entirely soluble in water, unless preserved by fixation in the histological sense. Moreover, in many cases, the precipitant fixatives alcohol and acetone, and mixtures containing them like Carnoy, fail to preserve the substrate from partial solution during the preparation of sections and they may allow its complete solution in buffer or water controls so that specific enzyme action is not demonstrable. In other cases alcoholic precipitation is satisfactory and leaves the substrate insoluble in buffers and in water. Nevertheless we are faced with the fact that choice of fixative has

usually been dictated by morphological considerations and salts of mercury and chromium, and even osmium tetroxide, have been used for preserving substrates for analysis by means of enzymes.

The chief considerations governing the choice of method for demonstrating the substrate before and after enzyme action are a relatively high specificity of the chosen stain for the substrate, and as close a relationship as possible between the colour developed and the amount of substrate present. If an ordinary staining technique is used it is essential that differentiation be avoided. Progressive staining should be carried out in very dilute solutions for a long period (24 hours) at a fairly constant temperature. The sections should then be examined after processes causing as little loss of stain as possible. Mounting in water is often the only satisfactory method since dehydration in alcohol may bring about violent differentiation and marked loss of stain. On the whole, it is preferable to take the more arduous step of employing a true histochemical reaction for some convenient grouping in the substrate, provided that the reaction chosen is free from the suspicion of removing substrate on its own account.

This general survey of the principles of the use of enzymes as histochemical reagents shows that methods based upon them are not entirely satisfactory. Even with numerous controls, it appears that the results achieved will be open to criticism. In the remainder of this chapter I propose to consider particular methods separately, taking first those which have been most used in modern histochemistry and which are, therefore, more important than the others. Two groups of enzymes are outstanding in this respect, the *nucleases* and the *hyaluronidases*. After them come two further groups of lesser importance, the *amylases* and the proteolytic enzymes *trypsin* and *pepsin*. Many other enzymes have been employed in analytical techniques, amongst them various *mucinases*, the *collagenases*, the *pectinases, lipases and lecithinases*, and finally enzyme batteries such as *Clostridium welchii* filtrates and snail stomach fluid ; they will be accorded brief mention here in view of their limited use.

The Nucleases

Ribonuclease

Nucleoproteins consist of basic proteins linked by salt-like union to nucleic acids. The protein part of the molecule is acted on by the proteolytic enzymes but it is not a substrate for the nucleases which we are considering here. It is thought that the nucleic acids are, in fact, broken down by a succession of enzymes, and these are divided into three groups, nucleases or nucleodepolymerases, nucleotidases (nucleophosphatases) and nucleosidases. The first of these, which

splits the nucleic acids into their component nucleotides, is now usually regarded as a pyrophosphatase (Baldwin, 1949). Greenstein (1944), however, regarded the nucleases solely as depolymerases. The second group of enzymes dephosphorylates the nucleotides to give nucleosides, which are glycosides of nitrogenous bases, and the third group breaks these down into bases and sugars. In histochemical analytical procedures we are only concerned with the first type of nuclease, whether its substrate be ribonucleic acid (RNA) as in the case we are considering, or deoxyribosenucleic acid (DNA) as in a following section.

The Use of Ribonuclease in Histochemistry. In 1913 van Herwerden showed that the basophilia of the chromidial granules in the sea-urchin oocyte, now known to be due to RNA, could be reversed by means of a crude extract of spleen, described as a nuclease. Little attention was given to this work. Ribonuclease was later described by Jones (1920) but no histochemical use was made of it until, following the observations of Dubos and MacLeod (1937) on the presence of an enzyme in purulent exudates which could remove the Gram-positive layer of pneumococci, Dubos and Thompson (1938) extracted from the same source an enzyme which specifically attacked RNA. Kunitz (1940) isolated the enzyme in crystalline form from ox pancreas and a great deal of the subsequent histochemical use of ribonuclease directly followed from his work. The purified enzyme is a soluble protein of the albumin type with a molecular weight of 15,000. It has its isoelectric point and its pH optimum at 7·7 and its optimum temperature at 65°. As a natural corollary, it possesses a high degree of thermostability and can withstand a period of 3 minutes in a water-bath at 90°, with trifling loss of activity. This property is made use of (see below) in order to free the enzyme from traces of proteolytic activity which remain in the majority of samples. Ribonuclease from different sources may vary in its properties (Ledoux and Brachet, 1955) and Kaplan and Heppel (1956) have recently described a ribonuclease from spleen and liver which differs considerably from pancreatic ribonuclease.

Much of the earlier work on the application of ribonuclease to fixed tissues gave results which, although they were sufficiently striking, were complicated by the fact that the impure samples of the enzyme which were employed possessed varying degrees of proteolytic activity. Even crystalline ribonuclease was found to have this proteolytic effect by Mazia (1941), and later Schneider (1946) confirmed these results and showed that different preparations of ribonuclease varied in proteolytic activity. With a view to determining the importance of this contaminating effect, Brachet and Shaver (1948) tested the effect of crystalline ribonuclease on protein structures using the tests described by Serra (1946) for arginine and

tyrosine. Tissues were fixed in an alcohol-formalin-acetic acid mixture and incubated for 1 hour at 52° with 1 mg./ml. ribonuclease in buffer at pH 7·0 or in water at pH 6·3. By the arginine and tyrosine methods no difference was observed between treated and untreated sections. It is certain, however, that neither of these methods gives a sufficiently sensitive qualitative appreciation for the conclusions derived from the above results to be considered valid (see Chapter V).

The earliest histochemical work on the detection of ribonucleic acids was that of Brachet (1940a and b), who showed that the cytoplasmic basophilia of cells fixed in Helly's fluid could be removed by treatment with a crude preparation of ribonuclease. He incubated sections for 1 hour at 65°–70° and subsequently stained them by the methyl-green pyronin method (see Chapter VIII, p. 205). Painter and Taylor (1942) incubated tissues for several hours with crystalline ribonuclease and stained them by the same method. Gersh and Bodian (1943) used crystalline ribonuclease at a concentration of 1 mg./ml. in veronal-acetate buffer at pH 6·75, incubating for 5 hours and subsequently staining with toluidine blue. They showed the basophilia of Nissl's granules in nerve cells was entirely lost after this treatment. Dempsey and Wislocki (1945) incubated sections fixed in Zenker-formol or Zenker-acetic for ½–3 hours at 60° and stained them by the eosin-methylene blue method. Dempsey and Singer (1946) used a similar method in investigating the basophilia of thyroid colloid and they used Mallory's trichrome stain and the Heidenhain azocarmine modification in addition to an eosin-methylene blue method. Deane (1946) used a 1 mg./ml. solution of ribonuclease, at pH 6·75 for 3 hours at 60°, on liver sections fixed in Zenker-formol and stained these with eosin-methylene blue. Stowell (1946) used the same strength of enzyme in McIlvaine's citric acid-disodium phosphate buffer at pH 7 and White (1947) used a 1 mg./ml. solution of a pancreatic extract whose proteolytic activity was first destroyed by boiling for 3 minutes in acid solution before buffering with a veronal-acetate mixture at pH 6·75. Sanders (1946) used a similar product in his studies on cytoplasmic nucleotides, destroying its proteolytic activity by heating at pH 6·75 and 80° for 10 minutes. Using his own technique and methyl green-pyronin staining, White studied the cytoplasmic basophilia of human marrow cells fixed in Heidenhain's Susa fixative, and by a similar method (Pearse, 1949) I studied the basophilia of plasma cells (human) and lymphocytes (guinea-pig) engaged in the production of Russell bodies and Kurloff bodies respectively.

In the majority of the above experiments control sections were incubated in water or buffer solutions for the same time and at the same temperature as the test sections. Stowell and Zorzoli (1947) drew attention to the fact that electrolytes in solution removed a

variable amount of RNA from control sections and observed that even distilled water had this effect at 60° and above. It is now customary, therefore, to use ribonuclease in glass-distilled water at 1 mg./ml. and 37°. Under these conditions the incubation period, although it varies considerably with the fixative, is about 1–3 hours. Using onion root tips fixed in acetic-alcohol, and incubating for 2 hours in the manner suggested above, Kaufmann, Gay and McDonald (1950) demonstrated an increased affinity of the chromosomes for acid dyes such as acid fuchsin. This was considered to be due to the release of a basic, tryptophan-containing, protein by removal of the nucleic acid previously attached to it. Using fixation by means of Flemming's or Navashin's fluids, Kaufmann, McDonald and Gay (1948) stained plant material with the safranin-gentian violet-orange G triple stain. They found that metaphase and anaphase chromosomes were bright red, whereas the chromonemata of late telophase and early prophase were violet. Ribonuclease was observed to remove the red-staining component, so that the metaphase and anaphase chromosomes appeared violet after hydrolysis. These findings showed that the crystalline ribonuclease used was free from protease activity, since the increased acidophilia of the chromosomes was not demonstrable, after even brief incubation, if protease was present as a contaminant.

The questions which remain to be answered are whether the use of ribonuclease is profitable in histochemical research and in diagnostic histochemistry, and what precise information can be derived from its action on fixed tissues?

The Specificity of Ribonuclease for RNA. In spite of earlier suggestions (Stowell and Zorzoli, 1947 ; Pollister *et al.*, 1950) that DNA was also affected by ribonuclease, more recent evidence indicates that this is not the case. McDonald and Kaufmann (1954) found that the reduction in Feulgen stainability of chromatin after ribonuclease was due to an intracellular deoxyribonuclease which hydrolysed DNA only after removal of RNA. If protease-free ribonuclease is used, in glass-distilled water and at 37° for short periods of incubation, material which is removed from fixed sections can be considered exclusively as RNA. Bearing in mind the objections discussed below, material which is not removed by such treatment is not RNA.

The Influence of Fixation on the Result and its Interpretation. Provided that the fixative employed does not cause loss of RNA, and does not render it impervious to the action of ribonuclease, it is possible to vary the incubation time so that removal of RNA from the tissues is complete. The question arises, however, as to how much the physical structure of the ribosenucleoprotein may alter its digestibility and how much alteration in this structure can be produced by the various fixatives. All authors agree in finding that fixatives

containing dichromates in acid solution (chromic acid) interfere dramatically with the enzymic removal of RNA. Such fixatives as Zenker, Zenker-formalin, Flemming and Navashin are not to be recommended, therefore, for ribonuclease studies. Inferior results which have been reported using Bouin, Schaudinn and methyl and ethyl alcohols in the absence of acetic acid, are largely due to imperfect preservation. These fixatives should also be avoided. Carnoy and Susa are strongly recommended by some authors (e.g. Kaufmann *et al.*, and White) and as strongly criticized by others (e.g. Stowell and Zorzoli). It is generally agreed, however, that formalin is a satisfactory fixative for RNA, and it can be used alone, or in combination with precipitant fixatives which do not interfere with subsequent enzyme action. Although mercuric salts may inhibit ribonuclease, the amount of mercury remaining in sections after treatment with iodine and thiosulphate is not sufficient to cause significant delay in removal of RNA by the enzyme. Carnoy, Susa, and formol-mercury mixtures are, therefore, recommended for fixation of tissue blocks and the first two for material in smears, which are destined for examination by the customary staining techniques after incubation with ribonuclease. Each of the three popular methods of demonstrating the action of ribonuclease has its adherents. A buffered eosin-methylene blue technique may be most suitable for widely distributed RNA, that of thyroid colloid, for instance. For marrow smears and most cytological work a buffered methyl green-pyronin stain gives the best results. The simplest, and often most effective method is simple and brief staining in dilute aqueous solutions of toluidine blue.

Only a brief survey of the literature is necessary before it becomes clear that information about the distribution of cytoplasmic nucleic acids has been derived from the use of ribonuclease which could not have been obtained by any other method, chemical analysis included. Therefore, none of the strictures recorded above should prevent an investigator from applying this particular example of modern histochemical analysis. If ribonuclease of satisfactory quality is not obtainable, the extraction methods listed and described in Chapter VIII and Appendix 8 may be employed as substitutes. A crude ribonuclease, free from amylase and protease, can be made by heating human saliva at 90° for 3–5 minutes, but whether critical work should be undertaken with this preparation is questionable.

Deoxyribonuclease

Much of what has been written above concerning ribonuclease refers equally to deoxyribonuclease (DNase), and it will not be repeated. Earlier histochemical work, using the latter enzyme, was carried out with preparations such as those described by Fischer *et al.* (1941)

and by McCarty (1946). Although these contained no appreciable amount of ribonuclease they were contaminated with varying amounts of protease. Later experimenters used a purer preparation, devised by Kunitz (1948), which was free from both impurities. The crystalline enzyme is activated by magnesium ions (optimum concentration 0·003M) and by manganese ions. The magnesium-activated enzyme is inhibited by 0·01M-citrate but manganese activation is unaltered. The enzyme is readily inactivated by heat and has its pH optimum in the range 6·8 to 8·2. Its isoelectric point is 5·1. Chargaff and Shapiro (1955) suggested that in the case of the pancreatic enzyme magnesium ions played a double part. After activating the enzyme, and causing the initial stages of degradation of DNA, magnesium ions are able to bring about additional, non-enzymic, breakdown of the initial reaction products. The results of the cytochemical application of purified DNase have not been uniformly satisfactory. Catcheside and Holmes (1947) used a Fischer-type spleen DNase, and a McCarty pancreas DNase, and produced complete abolition of basophilia and the Feulgen reaction in alcohol-fixed beanroot chromosomes by means of the latter. The splenic enzyme would achieve such results only after previous incubation of the material with ribonuclease. The authors believed that these results were due partly to impurities in their enzyme preparations and partly to the non-specific depolymerizing action of protein solutions. They advised caution in interpreting experimental results obtained with DNase.

Brachet and Shaver (1948) used DNase (Fischer) at a concentration of 10 mg./ml. and reported that the abolition of Feulgen staining and basophilia in alcohol-fixed chromosomes was "almost instantaneous" at room temperatures, provided that magnesium ions were present as activators. They used non-activated enzyme solutions for control sections. Using Ponceau 2R in acid solution as advocated by Hydén (1943) for the cytochemical detection of basic proteins of the histone type, these authors found a diminution in staining of the chromosomes after DNase which equalled the fall in basophilia demonstrated with toluidine blue. They remarked that this indicated either that Ponceau 2R stained DNA or else that DNase removed histones. The latter result was excluded by performing the arginine reaction of Serra, after DNase, and finding no diminution of staining intensity. In view of the known proteolytic activity of preparations of DNase of the Fischer type, it is difficult to understand this last result unless the particular Ponceau employed was capable of staining DNA in an acid medium. Brachet and Shaver found that their samples of Ponceau would in fact stain in this manner, although other authors (Hydén) could not stain histones with Ponceau 2R.

Vercauteren (1950a) found that a purified DNase (McCarty) whose

high activity against DNA *in vitro* was confirmed by a viscosimetric technique, failed to remove the basophilic material from chromosomes. Later (1950b) this author reported successful experiments using a crystalline DNase of Kunitz type. In these he confirmed the specificity of methyl green for DNA, already claimed by Pollister and Leuchtenberger (1949). He concluded that the DNase technique was dangerous as well as useful and that its results should always be compared with those afforded by other methods. A commercially available streptococcal deoxyribonuclease (Varidase, Streptodornase-Streptokinase, Lederle) was used by Jackson and Dessau (1954). These authors found that DNA was incompletely removed from sections treated with enzyme solutions containing less than 600 units per ml. Above this concentration complete removal was obtained but the authors did not investigate the specificity of the reaction. Kurnick (1955) reported that he obtained excellent results with a crystalline pancreatic deoxyribonuclease and it must be concluded that both this and the streptococcal enzyme can be used with some confidence. In an earlier paper (1954) Kurnick discussed the mechanism of DNA depolymerization by heat and showed that relatively mild treatment, insufficient to change its elementary composition, produced a reduction in affinity for deoxyribonuclease. This change was closely related to the accompanying fall in affinity for methyl green.

Hyaluronidases

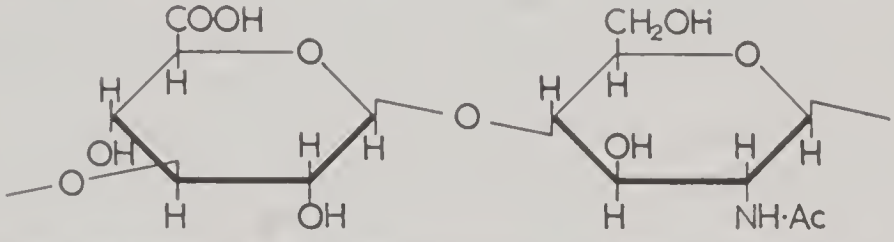
In recent years a number of enzymes have been obtained from various sources which are capable of depolymerizing and hydrolysing samples of hyaluronic acid and these are, therefore, referred to as hyaluronidases. A list of the sources of hyaluronidase appears in Table 48.

TABLE 48

Sources of Hyaluronidase

Source	References
Testis	Duran-Reynals (1928) ; McClean (1930)
Skin	Claude and Duran-Reynals (1934) ; Meyer *et al.* (1941)
Leech extract	Claude (1939)
Snake venoms	Chain and Duthie (1940) ; Favilli (1940)
Streptococci	Duran-Reynals (1933) ; Crowley (1944)
Staphylococci	Duran-Reynals (1933)
Pneumococci	McClean (1936) ; Meyer, Dubos and Smyth (1937)
Cl. welchii, septicum, œdematiens	Meyer, Hobby, Chaffee and Dawson (1940) ; McClean, Rogers and Williams (1943)
Schistosoma	Levine *et al.* (1948)

The history of the hyaluronidases begins with the description of the so-called "spreading factor," by Duran-Reynals (1928, 1929) and by McClean (1930, 1931), which was held to be responsible for the rate of diffusion of fluids in living tissues. The identity of this factor was discovered by Chain and Duthie (1939, 1940), who showed that the hyaluronidase activity of testicular extracts paralleled the activity of the "spreading factor." Meyer *et al.* (1936, 1937) had previously found, in autolysates of a rough Type II pneumococcus, an enzyme capable of hydrolysing hyaluronic acid. This substrate had been isolated shortly before by Meyer and Palmer (1936) from vitreous humour and umbilical cord. It is a protein-free polysaccharide, composed of glucuronic acid and *N*-acetylglucosamine in equimolecular proportions, which appears in the form of a high polymer.



Basic disaccharide unit of hyaluronic acid (Meyer and Fellig, 1950)

The average molecular weight of hyaluronic acid is between 200′000 and 500′000, and its solutions are characterized by high viscosity. Assuming a regular repeating unit (disaccharide) the distance between adjacent carboxyl groups would be 10Å.

Biochemistry of the Hyaluronidases

Tissue Substrates for the Hyaluronidases. It is necessary here to make a further brief reference to the terminology of the "mucins" which is employed in this book. This has already been considered in Chapter IX at some length. Confusion has occurred chiefly on account of the use of the term glycoprotein to mean mucopolysaccharide protein complex. Catchpole (1950), for instance, discussing components of the ground substance says, "assuming that one component, at least, of the connective tissue is a highly polymerized mucopolysaccharide or glycoprotein." This expression is ambiguous since, whatever the author's real view, it suggests identity between two classes. In the first Edition of this book and in published papers I followed closely the terminology put forward by Meyer (1938, 1945), in which the mucopolysaccharides were clearly distinguished from the mucoproteins and glycoproteins; in considering the substrates for hyaluronidase I propose to follow the same author. According to his view these substrates for the depolymerase fraction of hyaluronidase are all acid mucopolysaccharides, none are muco- or glycoproteins.

The latter may be substrates for the β-glucosaminidase of testis extracts in the same way as are the mucoprotein anterior pituitary hormones and chorionic gonadotrophins for mucinases produced by influenza virus and *Vibrio cholerœ* (Whitten, 1948).

As the result of prolonged studies on the component mucopolysaccharides of mesenchymal tissues Meyer and Rapport (1951) have been able to subdivide them into five types. These are hyaluronic acid, hyaluronosulphate, and chondroitin sulphate of three types, designated A, B and C. As substrate for hyaluronidase the second (hyaluronosulphate) which was found only in cornea, does not interest us very greatly, although it is rapidly depolymerized by both testis and pneumococcal hyaluronidase. The other types, however, are of widespread occurrence. Hyaluronic acid was found by Meyer and Rapport in synovial fluid, skin and umbilical cord. Chondroitin sulphate (Ch S-A) occurred exclusively in hyaline cartilage, Ch S-B in heart valves, aorta and tendon and Ch S-C in all the above sites except synovial fluid. The effects noted when these four compounds were tested *in vitro* with testis and pneumococcal hyaluronidases are condensed into tabular form below.

The data given in Table 49 repeat the statement of Meyer *et al.* (1941) that the testicular enzyme affects sulphate-containing and sulphate-free mucopolysaccharides while the bacterial enzyme affects particularly the latter. They would seem to suggest that by using hyaluronidase from the two sources mentioned we might be able to distinguish in the tissues four types of mucopolysaccharide-protein complex. This is not the case for two reasons. First, the above findings are based on hydrolysis and not on depolymerization, and the two functions do not run parallel. Secondly, they are based on material easily accessible to the enzyme. Whatever the theoretical considerations, the results given in the Table are not reproducible in the case of tissue sections, at least in those fixed in the usual formalin-containing or alcoholic fixatives. In the case of hyaline cartilage, for instance, neither the testicular nor the bacterial enzyme can remove the mucopolysaccharide component. The metachromatic substance of the aorta, the major part of which is composed of the hyaluronidase-stable ChS-B form according to Meyer and Rapport, behaves in a similarly paradoxical manner, resisting the bacterial enzyme as it should, but often being totally removed by testis extracts. The behaviour of the metachromatic substance(s) of skin, synovium and umbilical cord, which resists neither type of enzyme, is therefore histochemically correct. The metachromatic substance found in healing wounds has been stated by Bunting and White (1950) to be susceptible to testicular but not to streptococcal hyaluronidase. Penney and Balfour (1949), and Campani and Reggianini (1950) also removed the metachromatic

substance of non-scorbutic wounds by means of the testicular enzyme. Plate VIIIB illustrates the effect of the testis hyaluronidase on the metachromasy of normal connective tissue mucin. If unfixed tissues are used for histochemical tests with hyaluronidases the solubility of many of the mucopolysaccharide-protein complexes in water has to be taken into account. No difference may be recorded between removal by the enzyme and removal by the control solution.

TABLE 49

Actions of Hyaluronidases on Various Substrates

Substrate	Source	Hyaluronidase	
		Testis	Pneumococcal
Hyaluronic acid	Synovium Skin Umbilical cord	Labile	Labile
Ch S-A	Hyaline-cartilage Tendon	Labile	Labile
Ch S-B	Aorta Skin Tendon	Stable	Stable
Ch S-C	Aorta Umbilical cord	Labile	Stable

The pH Optima of the Hyaluronidases. The pH optimum for hyaluronidase varies not only according to the source of the enzyme but also according to the method by which it is estimated (see below).

Thus McClean (1943) found that the optimum pH values for the viscosity-reducing activities of testicular and *Clostridium welchii* hyaluronidases were 7·0 and 6·0 respectively. Rogers (1948) found the maximum viscosity-reducing activity of streptococcal hyaluronidase at pH 5·5 and of staphylococcal hyaluronidase at pH 6·6. As measured by release of reducing sugars the pH optima of the bacterial enzymes were, for the streptococcal variety, 5·6 and 7·1 (double optimum), for the staphylococcal variety 5·6, and for the *Cl. welchii* variety 5·7. According to Meyer *et al.* (1941) the optimum hydrolytic pH for testis hyaluronidase is 4·4 and for the pneumococcal enzyme, pH 5·6–5·8. These figures show that there is a wide variation in the optimum pH of all except *Cl. welchii* hyaluronidase depending on the method of assay which is employed.

Other factors which affect the activity of the hyaluronidases are also important from the histochemical point of view. The addition of salts, especially sodium chloride, has been observed to increase the activity of the enzyme from bacterial and mammalian tissue sources.

and this affects the viscosity-reducing far more than the hydrolytic activity. For testis hyaluronidase Madinaveitia and Quibell (1941) found the optimal concentration of NaCl to be between 0·07 and 0·17 M.

The Two Types of Hyaluronidase Activity. The action of hyaluronidases on hyaluronic acid is of two distinct forms (Meyer *et al.*, 1941), first a depolymerization which is rapid in action and, second, a slower hydrolytic reaction which liberates acetylglucosamine and glucuronic acid. Hydrolysis is estimated by testing the substrate solution for an increase in reducing substances, depolymerization by calculating the reduction in viscosity of the solution. Meyer (1947), in an extensive review of hyaluronic acid and hyaluronidase, suggested that two enzymes were necessary to break down the two types of glycosidic linkage in hyaluronic acid, one of which belongs to the *N*-acetylglucosamine and the other to the glucuronic acid (see formula on p. 229). The existence of two enzymes was also suggested by the observation that pneumococcal hyaluronidase would almost completely hydrolyse the substrate into its component monosaccharides, while testicular hyaluronidase hydrolysed only 50 per cent. in a comparable period. The latter, however, reduced the viscosity of substrate solution much more rapidly than the bacterial enzyme.

A similar conclusion to Meyer's was arrived at by Hahn (1945a and b, 1946a and b). Working with aqueous extracts of leech heads (1945a) he found that these caused only a 26 per cent. hydrolysis of hyaluronic acid, as measured by increase in reducing substances, while at the same time the reduction in viscosity was equal to that given by the best testicular preparations. In the case of the latter, he suggested that there were two enzymes present, one degrading hyaluronate to a disaccharide, lowering the viscosity and liberating half the total amount of reducing sugars, and the other hydrolysing the resulting *N*-acetylglucosamine glucuronide to its constituent monosaccharides. Rogers (1946a and b) showed that when purified hyaluronic acid was partially hydrolysed by enzymes prepared from three different sources, until about 50 per cent. of the theoretical amount of reducing sugar had been liberated, the size of the remaining units depended on the source of the enzyme. This suggested that the hyaluronidase enzyme system was even more complex than Meyer and Hahn believed. Rogers explained this observation by assuming that several enzymes successively degrade the polysaccharide to smaller and smaller units. He found that the optimal conditions of pH and salt concentration differed according to the enzyme preparation employed. Madinaveitia *et al.* (1940) considered that the hydrolytic and diffusing activities of testicular extracts were probably due to the presence of different entities, on account of large discrepancies between the results of two

different techniques for estimating the enzyme activity. McClean and Hale (1941) and McClean (1943), however, pointed out that these authors had performed the two tests under widely different conditions. Rogers (1948) measured the effects of streptococcal and staphylococcal hyaluronidases, by viscosity-reduction and reducing sugar methods, under identical conditions. The differences amounted to more than 100 times the standard error of the method and he concluded that the two methods did not measure the activity of one and the same enzyme.

As far as the aminopolysaccharase activities of the hyaluronidases are concerned there is general agreement that the testicular enzyme degrades hyaluronic acid and chondroitin sulphate (Types A and C) mainly to tetrasaccharides although disaccharides and a few higher oligosaccharides may be formed (Weissmann *et al.*, 1954; Schütte and Greiling, 1955; Hoffman *et al.*, 1956). Bacterial hyaluronidase, on the other hand, degrades hyaluronic acid to yield mainly a disaccharide (Schütte and Greiling, 1955; Linker *et al.*, 1956). Chondroitin sulphate A can be attacked by the bacterial enzyme if its ester sulphate groups are first removed by mild acid hydrolysis. Meyer *et al.* (1956) confirm that testicular hyaluronidase is without action on chondroitin sulphate B.

Histochemistry of the Hyaluronidases

Enzymes Available. Although the hyaluronidases are widespread in nature, in their application as histochemical reagents we are concerned only with two main sources, bacterial culture filtrates and mammalian testis extracts. The former are divided into (1) coccal filtrates and (2) clostridial filtrates. We have to consider the practical suitability of hyaluronidases from each of the available sources and the substrate or substrates in the tissues upon which they act.

From the first point of view there is no doubt about the greater convenience of testis extract, available commercially as a water-soluble powder of controlled potency, by comparison with the bacterial filtrates which must be freshly prepared in the first instance, although they are remarkably stable once prepared, even at room temperature. If the crude filtrates are used a number of enzymes besides hyaluronidase will certainly be present. *Clostridium welchii* Type A filtrates for instance, which have frequently been used as a source of hyaluronidase, contain also a so-called collagenase (κ-toxin), a lecithinase (η-toxin), and other lipolytic factors (lipases or esterases). Streptococcal and pneumococcal filtrates suffer from the same disadvantages, although the presence of other enzymes does not always matter. Whether their presence is important or not depends on which particular histochemical test is used for appreciation of hyaluronidase action on tissue sections (see below). Even testis extracts, in the crude form in which they

were formerly used for enzyme analysis, contained other enzymes besides hyaluronidase. Meyer and Linker (1951) reported that such extracts contained a β-glucuronidase and Houck and Pearce (1957) divide the mucolytic enzyme activities of ammonium sulphate-precipitated fractions of bovine testis into four groups. These are hyaluronidase, β-glucuronidase, β-hexosaminidase and β-galactosaminidase. The method of Dorfman (1955) is usually employed for the preparation of testis hyaluronidase for use in biochemical studies. The β-glucuronidase, however, is separable from the hyaluronidase fraction and in any case it is not present in the purified commercial preparations of testis which are used to-day.

The sole advantage of the bacterial (streptococcal or pneumococcal) enzymes, recently stressed by Davies (1952), is their specificity for the single substrate hyaluronic acid.

Staining Methods. The difficulties discussed above are accentuated by the fact that two very different methods have been employed for demonstrating the hyaluronidase substrates. The first method makes use of the metachromatic properties of the acid mucopolysaccharides, and toluidine blue is now the only dye used for this purpose. The second is the periodic acid-Schiff (PAS) technique. This was the sole method used by Gersh and Catchpole (1949) in a comprehensive study of ground substances and basement membranes. Before we proceed further it is necessary to emphasize the profound difference between the substances demonstrated by the two methods. According to Gersh and Catchpole the most characteristic component of the microscopically non-fibrillar ground substance is a glycoprotein (i.e. a protein containing a carbohydrate moiety as an integral part of its structure) and basement membrane is regarded as a closely related substance. The metachromatic component of ground substance, however, with which the pathologist is familiar as a bluish hæmatoxiphil material, is an acid mucopolysaccharide (hyaluronic acid or chondroitin sulphate). In the majority of situations neither staïns convincingly by the PAS method. Davies (1952) found that while an undegraded hyaluronic acid complex and a commercial preparation of potassium hyaluronate gave vigorous reactions in spot tests with the PAS reaction, an almost pure, sulphur-free, potassium hyaluronate gave a negative result. With toluidine blue all three produced metachromasia, labile to streptococcal hyaluronidase, but the PAS reaction of the first two was resistant to the enzyme.

Both the metachromatic mucopolysaccharide-protein component and the water-soluble glycoprotein component of Gersh and Catchpole are substrates for hyaluronidase but with this difference. The former was removed by short (3 hours) treatment with the enzyme, as evidenced by reversal of metachromatic staining. The latter was only

affected by prolonged treatment (20 hours or more at 37°), as shown by reversal of the positive PAS reaction.

Applications to Pathology. Most of the work so far reported in the pathological literature, in which hyaluronidase has been employed, describes the accumulation in the tissues of substances believed to be acid mucopolysaccharides. These have usually been demonstrated by metachromatic techniques, and hyaluronidases from various sources have been used to effect their removal, usually from conventionally-fixed paraffin-embedded material. The majority of workers have used testicular hyaluronidase and a lesser number streptococcal and clostridial filtrates. One report of the use of leech hyaluronidase has appeared (Negri and Weber, 1948), but this enzyme was found to be inactive against all the metachromatic ground substances tested. These authors reported that their crude testis extract was "extremely active against the chromotropic substance of blood vessel walls, causing total disappearance of the metachromatic coloration with toluidine blue." This finding is at variance with those of some other workers. Altschuler and Angevine (1949), for instance, using both crude and purified testis extracts, observed no activity against the metachromatic substance in arteries. Using relatively high concentrations of the testicular enzyme in saline, I find that arterial metachromasia is usually reversible by short treatment, in the case of both frozen and paraffin sections.

The earliest studies in the histochemical field were those of Mannozzi Torini (1942) who used a testicular extract which he described as a mesomucinase, to reverse the metachromasia of the ground substance of umbilical cord. Since this example, similar methods have been applied to the study of mucopolysaccharides in various normal and pathological tissues. Hyaluronidase-labile substances have been described, for instance, in rheumatoid nodules (Altschuler and Angevine, 1949; Fawns and Landells, 1953), in healing wounds (Penney and Balfour, 1949; Bunting and White, 1950), in Aschoff bodies (Bunting, 1950), in lupus erythematosus (Klemperer, 1948), in generalized and circumscribed myxœdema (Pearce and Watson, 1949; Watson and Pearce, 1950), in synovium (Asboe-Hanson, 1950), and in the cock's comb after treatment of the bird with pituitary hormones (Ludwig and Boas, 1950). These examples are only a few of many referring to the use of hyaluronidases; they are not, of course, the earliest references to the finding of metachromatic substances in the sites mentioned.

Conclusions. The conclusions which have been drawn by various authors as to the exact diagnostic significance of hyaluronidase-lability in a metachromatic mucopolysaccharide have tended to vary. Some have only made use of the observation to confirm the mucopoly-

saccharide nature of the substance investigated, and this is a valid if only partial conclusion if short incubation times are employed. Others have identified the labile substance with hyaluronic acid, an interpretation which may sometimes be true if testis enzyme is used, or wholly true, as in the case of streptococcal filtrates. At this present very imperfect stage of our knowledge of the nature of the substrates and actions of the hyaluronidases few conclusions can be considered accurate. I consider that metachromatic material, in fixed tissues embedded in paraffin, whose metachromasia is reversible by 1–3 hours' treatment with a purified testis extract (Rondase-Evans, Hyalase-Benger) : (1) is of mucopolysaccharide nature, and (2) is either chondroitin sulphate of Meyer's types A or C, or hyaluronic acid itself, or a mixture of any or all of these. Meyer himself (1947) was of the opinion that hyaluronic acid alone would not give rise to metachromasia in tissue sections, while chondroitin sulphates would do so. It is not possible to agree entirely with this view for the hyaluronidase-labile metachromasia of synovial tissues is apparently due to hyaluronic acid, the sole mucopolysaccharide constituent of synovium.

Chondroitinase

Certain strains of *Proteus vulgaris* have been shown by Dodgson *et al.* (1957) and by Dodgson and Lloyd (1958) to produce two enzymes, a chondroitinase and a chondrosulphatase, which together degrade cartilage chondroitin sulphate with the release of reducing substances and sulphuric acid respectively. The chondroitinase, however, can act independently of the sulphatase. Cartilage chondroitin sulphate is mainly type A with small amounts of type C ; type B is not present. The activity of their chondroitinase towards the latter has not yet been described by Dodgson and his associates and the enzyme has not been used histochemically. Hoffman *et al.* (1957) have described the production of adaptive enzymes in flavobacterium which would hydrolyse chondroitin sulphates A and C at high rates. Bacteria grown with β-heparin (identical with chondroitin sulphate B) as their carbon source produced an enzyme able to break the hexosaminidic bands to produce unsaturated uronides. These results have not been applied histochemically.

Other Polysaccharidases

Amylases

The enzyme amylase, as ptyalin in saliva, has been extensively used for the digestion of glycogen in tissue sections since its introduction for this purpose by Bauer (1933). The use of solutions of malt diastase, advocated by Lillie and Greco (1947), has now largely superseded the older practice. The preponderating hydrolytic enzyme of both ptyalin

and malt diastase is an α-amylase, belonging to the group which liquefy starch and glycogens in contradistinction to the more slowly acting β-amylases which exert only a saccharolytic activity against these substrates.

Neither of the two sources gives a pure α-amylase ; both are contaminated with other enzymes which may lead to various side effects. It has already been mentioned (p. 607) that saliva can be used as a source of ribonuclease and it has been shown by Lillie (1949) that malt diastase contains an enzyme which removes cytoplasmic basophilia. This enzyme resists a temperature of 90° at pH 3–4 and behaves in the characteristic manner of a ribonuclease. Lillie further observed that malt diastase solutions (often) had the property of destroying the metachromasia of nucleus pulposus and of cartilage matrix. Both the action on metachromasia and the ribonuclease action were to some extent dependent on the fixative used. They were both hindered by aqueous fixatives of the acid variety and by dichromate. It was not possible to obtain the metachromasia-destroying enzyme or the ribonuclease principle in separate fractions but the three enzymes could be distinguished by their differing resistance to certain procedures (Lillie, Greco and Laskey, 1949). The ribonuclease, as stated, resisted 90° at pH 3–4, the metachromasia-destroying enzyme resisted 60° at pH 3–4 and the amylase was destroyed by 60° at pH 6·0. Furthermore, it was observed that certain large Gram-positive bacilli present in the rabbit cæcum retained their Gram-positive staining, and their positive PAS reaction, after 4 hours' digestion with 0·1 per cent. malt diastase at 37°. When stained by means of Lillie's (1948) buffered azure-eosin technique, however, their staining changed from blue to pink. Precisely what action of diastase this indicates is not certain. It draws attention once more to the principle of using different final reactions to demonstrate the effect of any enzyme on tissue structures. In diagnostic histochemistry it is better to use only the best characterized demonstrative reaction (e.g. metachromasia in the case of the hyaluronidases), but research into less well understood actions of enzymes, making use of uncommon or little used reactions, will yield important results.

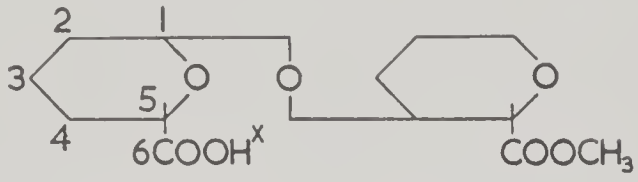
The above-mentioned side effects of ptyalin and diastase do not in any way influence the interpretation of results achieved by the use of saliva or malt diastase for removing glycogen from sections. With diastase it is unnecessary to use the enzyme either in buffer or saline, both of which have the tendency to increase its ribonuclease activity. Solutions in glass-distilled water remove glycogen adequately, even within as short a period as 30 minutes at room temperature, and if they are thus used little or no change will be produced in the tissue sections by the other enzymes present. The method of using either

ptyalin or diastase is given in Appendix 22. The choice of methods for demonstrating their glycogenolytic effect was discussed in Chapter IX, and the methods for this have been given in Appendix 9.

Pectinases

Biochemical Aspects. Enzymes of the pectinase group have been employed on acetone-fixed paraffin-embedded animal tissues by McManus and Saunders (1950). This group of enzymes was first described in 1898 in barley malt and they have since been shown to be widely distributed in fruits and to be formed by a number of bacteria and fungi. There are two main enzymes in the mixtures described as pectinase and the proportion of each varies widely, depending on the source.

The first enzyme, pectinesterase (PE), is particularly plentiful in plant sources such as tomato, potato leaves and tobacco. According to Lineweaver and Jansen (1951), PE hydrolyses methyl ester groups in polygalacturonides, if they are adjacent to free carboxyl groups, as at the $^{\times}$ in the diagram.



Methyl esters of polygalacturonic acid occur in long chains in the wide range of fruit pectins and these are the natural substrates for the de-esterifying action of PE. The enzyme is apparently highly specific (MacDonnell *et al.*, 1950) since it will not act on galacturonides but only on polygalacturonides. The second enzyme, polygalacturonase (PG), seldom occurs in higher plants but is widely distributed in bacteria and fungi. It also occurs in the animal kingdom having been found in snail-stomach fluid (Holden *et al.*, 1950). This enzyme acts on de-esterified portions of polygalacturonide chains, breaking the glycosidic links to give products of varying molecular weight.

Histochemical Aspects. From the histochemical point of view the situation would be much simpler than it is if the two enzymes described above were the only ones present in the various commercial and other extracts used under the comprehensive term pectinase. Unfortunately, a variety of different polysaccharidases is usually present.

McManus and Saunders (1950) investigated the activity of several pectinase preparations, and also a preparation which they described as a β-glucuronidase, using 0·4 per cent. solutions at pH 4 and 37° for 48 hours in each case. After incubation and brief washing, their sections were stained with hæmatoxylin and eosin and by the periodic acid-Schiff method. The authors noted that a loss of PAS-positive

material, mucin, glycogen, splenic reticulin and the ground substance of cartilage, occurred after the use of a preparation called pectinase (Nutritional Biochemical Company). A similar loss was observed after the use of polygalacturonase (Jansen and MacDonnell, 1945), and the same effect, to a lesser extent, occurred after the use of Pectinol O (Rohm and Hass). Pectin esterase (Rohm and Hass) was noted to increase rather than decrease the PAS reaction of the tissues listed. β-glucuronidase was without action, while diastase at pH 6·8 removed "everything" from the sections. The authors divided the actions of their preparation called pectinase, which must presumably be regarded as a polygalacturonase, into two groups. The first was a morphological action, removing such materials as "hyaline" while leaving elastica and nuclei untouched, which brought about a kind of microdissection on the slide. The second was a purely histochemical action. Their "morphological" action is, in fact, an employment of enzymal analysis in the old sense where the enzyme is used as a macerating agent. They believed that the evidence that PAS-positive materials were carbohydrates was enhanced by their removal of pectinase, and concluded that, in the future, pure enzymes of the pectinase group might give chemical information about tissue structures composed of or containing the appropriate substrate. McManus and Cason (1951) showed that the removal of PAS-positive material by pectinase was prevented by prior acetylation (Appendix 9, p. 832). This was regarded as additional evidence in favour of the action of pectinase on a carbohydrate substrate. The results obtained by McManus and Saunders in acetone-fixed tissues were duplicated by Cambel (1952) with ethanol-fixed mucin-containing tissues, and by Eidinger and Ghosh (1956) using acetone. The latter authors agreed that pectinesterase intensified the PAS reaction of collagenous tissues but found this effect with pectinase also. Eidinger and Ghosh also examined the effect of the two enzymes on tissues fixed in Bouin, Helly and Carnoy's fluids. In this case, they noted little loss of staining but an intensification with both enzymes in a number of sites which were usually PAS-negative or only weakly positive. Boiled enzyme solutions did not have any effect on removal of tissue components, but some of the increased staining was observed to be due to adsorption of carbohydrate components of the enzyme on to tissue proteins. This was prevented by prior acetylation. They considered that an unknown mucinase, present in commercial pectinase preparations, must be responsible for removing carbohydrate components from acetone-fixed tissues.

These propositions must be examined. First of all, we can omit the further consideration of pectinesterase for two reasons. This enzyme should not remove any naturally-occurring substrate from animal tissues, and McManus and Saunders observed that it indeed had no

such action on the tissues which they used. When we come to examine the case of polygalacturonase we have to decide two points, the interpretation of the removal of PAS-positive substances from animal tissues by crude extracts containing the enzyme, and the probable interpretation if pure polygalacturonase had been employed with similar results.

According to Lineweaver *et al.* (1949), PG is highly specific by comparison with the simple glycosidases from which it can be differentiated by its failure to hydrolyse methyl-α-D-galacturonic acid. Neither crude nor purified PG attacked gum ghatti, pneumococcus Type I polysaccharide, heparin or hyaluronic acid. The first two of these contain 12 and 60 per cent. respectively, of galacturonic acid, while the other two are glucuronides. The polymannuronide alginic acid, now quite often found in sections from operation sites where calcium alginate has been used for hæmostasis and packing, is similarly impervious to crude or purified PG. The crude preparations, however, contain enzymes which can hydrolyse starch, maltose, sucrose, carboxymethylcellulose, inulin and other glycosides. It is probable that some of these enzymes act upon the glycosidic linkages of hyaluronic acid and chondroitin sulphate. These are both glucuronides but differ in that the former contains *N*-acetylglucosamine and β-glucosidic linkages while the latter possesses *N*-acetylgalactosamine and β-galactosidic linkages.

No definite conclusion can be drawn from the reversal of the PAS reaction in animal tissues by crude PG preparations, except that the likelihood of labile components being polysaccharide is increased. If a " pure " PG becomes available, and if this continues to remove PAS-positive substances from animal tissues, it will be necessary to conclude either that it is not a pure enzyme at all (if it is true that no substrate for PG is normally present in such tissues), or that PG acts on linkages in polysaccharides which have not yet been described. For the diagnostic histochemist, therefore, the use of either crude or purified preparations of pectinase is unlikely at present to yield any information of an exact nature, and if the component being studied is, in fact, removed by a pectinase preparation the picture may well be complicated instead of the reverse. In spite of these strictures, details of the use of pectinase are given in the Appendix to this chapter.

β-glucosidases

There are two convenient sources of enzymes belonging to the class of β-glucosidases ; these are the so-called emulsins, derived from *Aspergillus oryzæ* (taka-diastase) and from the digestive tracts of various snails, especially *Helix pomatia*. The term " emulsin " means

a crude or partially purified mixture of β-glucosidases derived from any source, and there are numerous other enzymes present in both the above-mentioned examples. Neither has been much used in histochemical research but the second, under the heading of snail-stomach fluid, has perhaps received more attention than the first. It is the reddish-brown liquid from the crop of a starved snail. More than thirty enzymes have been detected in this fluid (Holden and Tracey, 1950) and of this number two-thirds belong to the class of carbohydrases.

Snail-stomach fluid was recommended by Fabergé (1945) as a reagent for use in plant cytology and its use for plant materials was discussed by Chambers (1955). It has been used by Gersh (1949) in his studies on the Golgi apparatus in mammalian duodenal cells. He found that 1 hour's incubation reduced the PAS reaction of the apparatus to vanishing point. This finding Gersh interpreted as indicating the presence of glycoprotein since he regarded the chief action of snail-stomach fluid as a depolymerization of polysaccharides. Gersh and Catchpole (1949) also used the fluid and found that, with 1 hour's incubation, it had no effect on the glycoproteins of ground substance and basement membranes which they were studying. They rightly ignored this result. We are faced with the question of deciding what substrates in animal tissues are attacked by the battery of enzymes present in snail-stomach fluid. Holden and Tracey have shown that hyaluronic acid and alginic acid are resistant to snail-stomach fluid and there is no reason to suppose that chondroitin sulphate would behave differently. Glycogen is certainly attacked, but this substance is easily distinguishable, and fatty materials may be broken down by a lipase which is present. These are also distinguishable, by parallel tests, from the purely protein constituents of any given tissue. It is probable that the views of Gersh are correct and that any reduction of the PAS-positive reaction of a tissue structure by snail-stomach fluid can be taken to indicate the presence of carbohydrate.

The use of snail-stomach fluid is scarcely warranted in the ordinary diagnostic histochemistry of animal tissues. In the case of plant tissues it may be useful as a source of cellulase.

Acetylglucosaminases

A number of crude extracts containing chitinases have been described (Zechmeister and Tóth, 1939) and enzymes of this type are present in bacteria, molds and *Actinomyces*. Recently the preparation of a much purer chitinase has been described by Jeuniaux (1957). This enzyme, obtained from *Streptomyces* sp. has an activity 70 times higher than that of the original product. Purified chitinases have obvious uses in the histochemical study of developing chitins and for

investigating the large number of reputedly chitinous tissue components which fail to react in a satisfactory manner to histochemical tests.

The Proteinases

Trypsin, Chymotrypsin and Pepsin

The three main proteolytic enzymes are here dealt with in a single section although their histochemical actions are not entirely similar. All three have been prepared in crystalline form, pepsin by Northrop (1930), chymotrypsin by Kunitz and Northrop (1935), and trypsin by McDonald and Kunitz (1946), so that they are available in a high degree of purity. These three enzymes are all described as endopeptidases, since they can act not only upon the peptide links of terminal amino-acids in the protein chain, like the exopeptidases, but also on similar links within the chain. Their specificities for these internal links are somewhat different and it is worthwhile recording them although, at present, their translation into differential histochemical diagnosis is not possible.

Trypsin acts upon peptide links incorporating the carboxyl (COOH) groups of arginine and lysine, chymotrypsin on those formed from the carboxyl group of an aromatic amino-acid, and pepsin on the link between the α-carboxyl of a dicarboxylic amino-acid (e.g. aspartic, glutamic) with the amino group of an aromatic amino-acid. Because of these different specificities mixtures of the three enzymes will break down a greater number of peptide links than any one of the three acting separately.

A histochemical interpretation of the actions of trypsin and pepsin is suggested by the work of Mazia *et al.* (1947) and of Kaufmann *et al.* (1950) on the protein structure of the chromosomes. The former authors showed that trypsin, as expected, was able to digest both histone (basic) and non-histone proteins although they believed that the main histochemical action of trypsin was attributable to the digestion of peptide links in basic amino-acids. These would be more numerous in histones than in more acid proteins. Kaufmann *et al.* agreed substantially with these findings and observed no final difference in sections treated with trypsin and those treated with chymotrypsin. They showed, furthermore, that in the absence of electrolytes solutions of either enzyme (0·1 mg./ml. in water at pH 6) failed to digest proteins sufficiently to cause their removal from the sections. (They did, however, have an effect in increasing the affinity of the chromosomes for pyronin which was attributed to some degradation of ribonucleoprotein.) If these trypsin-digested sections were subsequently placed in buffer or sodium chloride solutions, full digestion of their protein components took place and the protein could then be completely

removed by washing with water. Kaufmann *et al.* therefore declared that the histochemical action of trypsin was largely attributable to the removal by water of the enzymic digestion products after their further degradation by various salts.

Using salivary gland chromosomes as his test object Mazia (1941) showed that solutions of pepsin in HCl caused only shrinkage and no impairment of structure. He believed that this shrinkage was due to removal of a " matrix protein " containing many acidic groups. Mirsky (1943), however, suggested that it was equally likely to be due to the breakdown of histones to histopeptones. The problem resolves itself into whether histones as well as non-histones are removed by treatment with pepsin. Mazia *et al.* (1947) endeavoured to answer this question. They found that pepsin would not digest histones or combined histones (nucleohistones) in solution, but that it would digest more acidic proteins under similar conditions. These findings, unfortunately, are not exactly applicable to the proteins of fixed tissue sections since wide variations can be caused by the chemical and physical assaults suffered during fixation and embedding (see Chapter IV). Kaufmann *et al.* believe that the principal action of pepsin, recognizable histochemically, is the removal of tryptophan-containing proteins of the non-histone variety. Their findings in the case of chromosomes (onion root) suggest that such proteins are present in considerable amounts, and this agrees with the views of Pollister and Leuchtenberger (1949) though not with the values derived from chemical analysis of isolated nuclei (which ought to be more reliable).

Pepsin and trypsin differ in their action on native collagen fibres in the test-tube, only pepsin having any marked digestive effect at temperatures below 40°, but fixed collagen in tissue sections is digestible by both enzymes though relatively faster by pepsin. The difference in the digestibility of reticulin and collagen, already referred to in Chapter VII, is not of much practical importance since distinction between the two is more readily made by means of silver methods of staining. Tryptic digestion has been used even by modern authors for the purpose of indicating whether a tissue component is or is not composed of protein. Gersh (1949) thus recorded the protein nature of the Golgi apparatus in mammalian duodenal cells and Day (1947) established the fact that the amorphous ground substance between collagen fibres also contained protein. We may conclude from all the above studies that trypsin and pepsin can be used histochemically either for the simple confirmation of the presence of protein or for the more important purpose of obtaining information upon the nature of the protein in a given structure.

Benditt and French (1953), in a study of the effects of various enzyme preparations on cartilage considered that the reduction or

abolition of a specific staining method after treatment of sections with protein-containing solutions might be due to four activities: (1) depolymerization (specific or non-specific), (2) proteolysis, (3) masking of reactive groups by protein binding, and (4) displacement without depolymerization. The presence of traces of proteolytic activity in many depolymerase preparations is the most common source of confusion. The other contingencies mentioned by Benditt and French should be borne in mind, however.

Papain

From the mixture of proteolytic enzymes found in extracts of the latex of *Carica papaya* (paw-paw), which is called crude papain, two peptidases have been isolated in crystalline form. Besides these two a number of other enzymes exist in the crude extract and this makes the use of the latter as a histochemical reagent a source of difficulty in interpretation of results. Nevertheless, some of the biochemical work referred to below suggests the possibility of using even the crude extract in diagnostic histochemical procedures. Middlebrook and Phillips (1941) showed that the keratin of wool fibres was rapidly attacked by papain in the presence of sodium bisulphite at pH 6·7 and this action was further investigated by Blackburn (1950) who discussed the whole question of keratins and enzymic digestion. While intact keratins are resistant to the action of proteolytic enzymes such as trypsin, pepsin and papain, keratins which have been reduced —i.e. in which the disulphide groups have been converted into thiols— are readily attacked. Blackburn stated that sodium bisulphite at pH 6 or 7 markedly increased the digestion of keratin by trypsin, but in this case, and after digestion of wool by papain, histological examination of the residues indicated that the cortical cells were still resistant. This finding would appear to limit the usefulness of the above for histochemical application and no such application appears yet to have been made. Blumberg and Ogston (1958) used a crystalline preparation of papain for removing protein from the protein complexes of hyaluronic acid. They suggested that in rheumatoid arthritis the protein part of the complex is abnormal.

Elastase

An elastolytic enzyme was discovered in bacterial culture filtrates by Eijkman (1904). The most active organism in this respect was *Ps. pyocyanea*. The preparation from pancreas of an enzyme which specifically attacks elastic fibres was described by Baló and Banga (1949, 1950). These authors used their preparation to digest elastic fibres in histological sections of aorta and they showed that the enzyme had no action upon collagen fibres from tendo Achillis, although it

caused complete removal of elastic tissue. The first step in this process was described as a swelling, followed by breaking of the fibres into small particles, and subsequently by their complete solution. Since no amino-acids were liberated from elastin when this was acted upon by the enzyme the authors considered that its elastolytic activity depended on the conversion of the " long rod-shaped " molecules of elastic into molecules of a globular type. The pH optimum of the enzyme was in the region of 10·3. Banga and Schuler (1953) concluded that elastin was a glycoprotein since they were able to demonstrate carbohydrate in elastic tissue after depolymerization by elastase. Pepler and Brandt (1954) compared the activity of a bacterial chondrosulphatase obtained from *Ps. pyocyanea* and from *P. vulgaris* with bovine pancreatic elastase. With short incubation both types of enzyme had an identical chondrosulpholytic effect and with longer incubation an identical elastolytic effect. The authors maintained, therefore, that elastase could be regarded as a chondrosulphatase. Banga and Baló (1956) now regard elastase as inhomogeneous. Their preparations contain a mucolytic enzyme in addition to the elastase which acts on serum mucoproteins and on the carbohydrate-containing protein of native and fixed collagen. Lewis *et al.* (1956) describe the preparation of a purified elastase, together with its properties, and Tennant *et al.* (1956) describe the application of such a preparation to experimental atherosclerosis.

Collagenase and Reticulinase

After the toxin found in *Clostridium welchii* Type A filtrates had been shown by Macfarlane and MacLennan (1945) to produce necrosis in rabbit muscle, the associated histological picture was described by Robb-Smith (1945). This author observed the disappearance of the reticulin membrane of the muscle fibre and the intermysial connective tissue and ascribed these to the action of a collagenase. Dresner and Schubert (1955) applied a purified clostridial collagenase of this type to a number of collagen and reticulin preparations *in vitro*. All were readily hydrolysed by the enzyme with the release of hydroxyproline-containing peptides. Bidwell (1949) reported her findings on the nature of the collagenase activity of *Cl. welchii* filtrates. She suggested that a second collagenase could be produced in the filtrates either by heating to 50° for 10 minutes or by altering the pH to 9·0 or 10·0. This second enzyme was considered to be derived from an inactive precursor, or from the original enzyme. Many authors disagreed with Robb-Smith both in respect of his observations and of his interpretation of these. Gersh and Catchpole (1949), for instance, used a purified κ-toxin (collagenase) and found it inactive against purified collagen or against collagen in tissue sections ; it was capable

of removing the glycoproteins of the ground substance, however. They concluded that the term collagenase was inappropriate. Aikat and Dible (1950), using Type A filtrates, could find no evidence of collagenase activity (i.e. destruction of collagen) but they did find strong evidence of what was thought to be a hyaluronidase effect in that the collagen bundles tended to be broken down into their constituent reticulin fibrils. It was believed that the cement substance binding the fibrils together contained hyaluronic acid.

Robb-Smith (1953) reviewed the literature on proteolytic enzyme systems derived from clostridial filtrates. Some of these are certainly collagenases and gelatinases, broadly speaking. Ogle and Logan (1956), for instance, have shown that the "gelatinase" of *Cl. histolyticum* contains at least two metal-containing peptidases, inactivated by versene, in addition to many others. My conclusion, in the first edition of this book, that clostridial enzymes could not be regarded as collagenases was perhaps an overstatement. To redress the balance I quote Robb-Smith (1953) for the final word. "In using collagenase one must never forget that as yet it is not available in anything like a pure state and one has no real idea what other enzymes may be present in a bacterial filtrate." "Further, if any significance is to be attached to reactions of enzymes of this class, great care must be taken to ensure that the collagen or reputed collagen has not been denatured in any way." "Nevertheless, with these limitations, and if care is taken to apply proper controls, collagenase can be of value in the analysis of connective tissue fibrils."

Lipase and Lecithinase

Lipases have found little use in histochemistry but a small amount of work has been done with the various lecithinases. The usual substrate for these is lecithin but there is evidence (Macfarlane, 1942) that sphingomyelins are also split. There are four types of lecithinase (A, B, C and D). The first liberates one only of the two esterified fatty acids to give a product known as lysolecithin. To this product the snake venoms owe some of their toxic character. Lecithinase B liberates the two fatty acids and lecithinase C liberates choline only, leaving a phosphatidic acid. Lecithinase D splits lecithin into two fragments, a diglyceride and phosphoryl choline.

The α-toxin of *Cl. welchii* owes its activity to the presence of a lecithinase, as shown originally by Macfarlane and Knight (1941) who found that when α-toxin split lecithin into phosphoryl choline and a diglyceride the presence of Ca or Mg ions is necessary.

Lecithinase C activity is also apparently present in *Cl. welchii* α-toxin and this was used by Nygaard *et al.* (1954) in their studies on the effect of these enzymes on mitochondria. Morrison and Zamecnik

(1950) used snake venom and bacterial lecithases on tissues and found that fat appeared (demonstrable with Oil red O) after incubation with the snake venoms only.

Only unfixed tissues are suitable for lecithinase studies and *Cl. welchii* α-toxin is the reagent of choice. It is usual to incubate thin blocks at 37° for 12–18 hours and then to fix the tissues in formalin before cutting sections and using fat stains. Materials capable of taking up the solvent fat stains, such as Oil red O are produced by the action of lecithinase on a variety of tissues. Interpretation of the results, which often have a rather dirty appearance, is far from easy.

The action of a lecithinase (phospholipase) A from the dried venom of the cottonmouth moccasin *Agkistrodon piscivorus piscivorus* on ovolecithin has been described by Long and Penny (1957). Its activity is stimulated by Ca^{2+} ions between 40 and 80 μM when the substrate concentration is 1·3 to 3·3 mM. and EDTA (ethylenediamine tetraacetic acid) acts, not unnaturally, as a potent inhibitor of the activity of this type of lecithinase. Davidson and Long (1957) carried out a survey of plant tissues in search of a strong source of phospholipase D, capable of liberating choline from the naturally occurring phospholipids. The highest activity was present in the leaves of the Savoy cabbage and in Brussels sprouts. An associated phosphodiesterase activity could be separated from the lecithinase activity by adsorption on $Ca_3(PO_4)_2$ gel.

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CHAPTER XXIII

PIGMENTS

Introduction

In this chapter the following substances will be considered : melanins, adrenalin, argentaffin (enterochromaffin) granules, lipo- and hæmo-fuscins, lipochromes, hæmoglobin and porphyrins, hæmosiderin and bilirubin or hæmatoidin. Theoretically, this arrangement is indefensible. Each member of the group could more logically be considered elsewhere : thus melanin and hæmoglobin should appear in Chapter V as chromoproteins, and the lipid pigments might be dealt with in Chapter XI. Hæmosiderin might logically be considered in Chapter XXIV, where the methods for its demonstration are described. In practice, however, histologists and cytologists very often find pigmented substances in the sections they are examining and wish to identify them. This chapter collects together all the important naturally-occurring pigmented substances, or their precursors, and considers their histochemical identification. The practical value of this procedure outweighs any theoretical disadvantages.

The chapter falls naturally into three divisions : (A) *Tyrosine-Tryptophan pigments*—melanin, adrenochromes, argentaffines. (B) *Hæm pigments.* (C) *Lipid-pigments.* All the substances to be dealt with can be classified as one of these three.

The Tyrosine Group

The Melanins

The word melanin is derived from the Greek *μέλας*, meaning black, but it is used to refer to a group of pigments which may be yellow, brown, black or even violet. The intestinal pseudo-melanins are considered in the section on lipofuscins. The process by which the melanin pigments are thought to be elaborated from tyrosine or tyrosine-containing precursors has already been described in Chapter XVIII, in the section dealing with tyrosinase (DOPA oxidase). It is not known whether tyrosine itself is the precursor of melanin in the mammalian organism but, at all events, brown or black pigments can be produced *in vitro* by the oxidation of dihydroxyphenyl compounds and the subsequent polymerization of the products into substances of high molecular weight. The biochemical basis of human melanin pigmentation has recently been discussed by Fitzpatrick and Lerner (1954) and Mason (1955) has reviewed the matter from the

enzymologieal point of view. The association of melanin with protein is an important one which is responsible for mueh of the diffieulty in extraeting the pigment from the tissues. It is also responsible for differenees observed between natural and synthetic melanins (Stein, 1954). Gortner (1912) deseribed a melanoprotein in wool and Greenstein *et al.* (1940) obtained a water-soluble melanin-eontaining pseudoglobulin from S91 mouse melanoma. Mason *et al.* (1947) subjeeted this water-soluble pseudoglobulin, and also melanin granules from the same souree, to electron mieroscopy. They found that the two were identical and consisted of regular round or rod-shaped bodies, 0·2 μ in average diameter, which were aggregated in ammonium sulphate and redispersed by its dialytic removal. This is to say, they behaved as a pseudoglobulin.

It is still possible that melano-proteins may be formed by the oxidation of tyrosyl radicals in a protein ehain, rather than from free tyrosine with later eombination with protein. In this eontext it is important to note that Sizer (1947, 1948) has reported that mushroom tyrosinase can oxidize tyrosine present in the peptide chain of proteins. However, mammalian tyrosinase probably eannot aet in this manner since it cannot oxidize tyrosine if the amino group is combined with acetyl or formyl radicals.

The Structure of the Melanin Granule. The problem of the presence or absenee of a earrier, in the form of a protein nueleus to the melanin granule has been eonsidered extensively by Lignae and his associates (Lignac *et al.*, 1952 ; Lignac, 1954) who eonelude that there is a residuum remaining after bleaehing whieh ean be so regarded. The problem of the development of the melanin granule from mitoehondria has been examined by Stein (1955) by means of eomparative ehemical studies. He shows that the differenees between the two are so considerable that it is unlikely that melanin granules are in fact derived from mitoehondria.

Histochemical Characteristics of the Melanins

Recognition of melanin in the skin or the eye is not in praetiee a matter of difficulty. Difficulties arise with unidentified brown pigments in, for instance, the bowel wall or in secondary tumours of uncertain origin. It is here that the few positive reaetions of melanin, usually regarded as typieal, are in faet a souree of eonfusion sinee they are shared with other types of pigment (see Table 50, p. 673).

Solubilities. The most striking physieal characteristie of the melanins, from the point of view of the histologist, is their eomplete insolubility in organie solvents or in anything which is not markedly destruetive towards the tissue preparations which contain them. Lea (1945) stated that melanin was soluble in ethylene ehlorohydrin,

but this solvent and others mentioned by Mason (1948a) are said to be satisfactory only after the pigment has been separated from its protein component. Taft (1949) treated fixed and unfixed sections of skin with ethylene chlorohydrin and with pyridine, at 22° and 66°, for periods of 15 minutes to 24 hours. He could find no diminution in the amount of melanin in any of the sections. It is often stated that melanin granules are soluble in strong alkali (NaOH, KOH) if this is applied for a sufficient period of time, and that lipofuscin is not. This feature is not much used in histochemical diagnosis, although it might be if an unknown pigment was thought to be either melanin or one of the lipofuscins. (The solubility in fat solvents of the latter, even when unfixed, is not comparable to that of glycerol esters or phospholipids, so that no useful purpose is served by testing with various extractives.)

Bleaching. The second characteristic of the melanins is the facility with which they are bleached by strong oxidizing agents such as H_2O_2, acid chlorate, $KMnO_4$ or $FeCl_3$, although the process is always a slow one. The blacker the melanin the slower its decolorization. Figge (1939, 1940), reported that sodium hydrosulphite ($Na_2S_2O_4$, dithionite) or ascorbic acid would convert a black melanin from the ink sac of the squid, or that produced by the *in vitro* oxidation of DOPA, to a light tan colour. On addition of potassium ferricyanide the black colour was restored. There seems to be some doubt about the reversibility of decolorization since the brown, yellow and finally colourless melanin produced in tissue sections by any of the four strong oxidizing agents mentioned above cannot be restored by ferricyanide. After decolorization, melanin granules in human skin and melanomata cannot be shown to contain tyrosine either by Millon's or by the coupled tetrazonium reaction. They can be demonstrated, however, by means of silver reactions employing a reducing agent. At least 24 hours' treatment is necessary to decolorize melanin as a rule; 10 per cent. H_2O_2 is a most convenient and satisfactory reagent in my experience (see Appendix 23), but stronger solutions often have to be employed. Performic and peracetic acids also bleach most melanins very rapidly (Lillie, 1956) and the second, being commercially available is the more convenient of the two. Melanin in paraffin sections is more easily bleached than melanin in frozen sections, from identical sites.

Other Reactions of Use in Identification. Another characteristic of the melanins is their ability to reduce solutions of ammoniacal silver nitrate to metallic silver. This property was first made use of for their demonstration in tissues by Bizzozero (1908). Masson's (1914) method for their demonstration, which also depends on this property, has been universally employed for the purpose, in many modifications. Melanins are also blackened by acid silver nitrate

solutions, as observed by Giroud *et al.* (1934) and by Clara (1942). This phenomenon has recently been considered by Lillie *et al.* (1957) who have shown that at pH 4·0 relatively few substances reduce $AgNO_3$ in 10 minutes or less. Of naturally occurring substances likely to be present in tissue sections only two, ascorbic acid and *o*-diphenols, performed in this manner. I have used either Gomori's (1948) hexamine-silver method, recommended for the demonstration of melanin by Lennox (1949), or the more usual Masson-Fontana solutions ; details of both appear in Appendix 23.

Besides being argentaffin, melanin granules are also argyrophil, using this word to mean that silver impregnation methods employing a reducing agent colour them black. This reaction is not of diagnostic value. Many of the basic dyes colour melanin strongly, especially methylene blue and toluidine blue in acid solutions, but this property is relatively useless since the lipid pigments are often just as basophilic when stained with the dyes mentioned. Lillie (1955) made some tests of this property and observed that the basophilia of melanin was a true basophilia and not a false one as had been supposed by Hueck (1912). Since melanins can be stained green with thionin at pH 1·0 and since at this pH the only other tissue components which stain are those containing sulphonic acid groups or ester sulphates, Lillie suggested that these were responsible for the basophilia of melanin. Sulphur has long been known to be present in melanin and it is possible that oxidation of S-S groups to SO_3H can occur under natural conditions. The reduction of ferricyanide to ferrocyanide and the production of Prussian blue in the presence of ferric salts (the so-called Schmorl's reaction, see below), is shared by the lipid pigments and by argentaffin cell granules. One can nearly always say that a pigment insoluble in anything which leaves the tissues intact, but soluble in N NaOH, which is bleached by strong oxidizers in less than 48 hours, and which reduces ammoniacal silver nitrate, belongs to the class of melanins. In my opinion Schmorl's reaction is superior to the ammoniacal silver techniques for demonstrating melanin, especially the finer grades of melanin and minute granules of promelanin, which do not reduce silver salts. Figs. 200 and 201 illustrate the difference between the two techniques, in serial sections of the same tumour.

Ferrous Ion Uptake. A reaction which is apparently specific for melanin was described by Lillie (1957a) who found that melanins form complexes with Fe^{++} which can be demonstrated by means of potassium ferricyanide (Turnbull blue reaction). Ferrous ion uptake, demonstrated in this way, differs from Schmorl's reaction in that there is no reduction of Fe^{+++} to Fe^{++}. As Lillie points out, *preliminary* treatment of melanin with $FeCl_3$ results in a positive Turnbull blue reaction when ferricyanide is applied later. This is not true of

other components which give a positive Schmorl's reaction. With these it is necessary to apply the reagents simultaneously. Thus it appears that some part of the melanin complex is able to chelate Fe^{++} and Lillie suggests that this is an *o*-quinhydrone configuration. Details of this technique, whose specificity for melanin is very high, are given in Appendix 23, p. 919.

Reactions Used for Determining the Structure of Melanin. Although according to Mason (1948b) melanin is a polymerized indole derivative, Lea (1949) stated that Ehrlich's *p*-dimethylaminobenzaldehyde test for indole was negative with fully formed melanin. He found it positive for a melanin precursor which he described as melanogen. These melanin precursors are not demonstrable in melanoblasts by means of the above reaction, and melanin in tissue sections always gives a negative result. McManus (1949) stated that melanin under certain conditions contains carbohydrate, an interpretation based on the occasionally positive PAS reaction which it gives. This reaction, in my experience, is only positive with melanin in melanophages (phagocytes) and it is always negative in melanoblasts and epithelial cells. What significance the positive reaction has is not clear. I do not consider it indicates a carbohydrate component, but that either (1) the melanin is being broken down in such a way that mild oxidation (HIO_4) reveals aldehydes formed from the pigment itself, or (2) a coating of oxidizable unsaturated lipid surrounds the pigment. The second is the more likely of these two hypotheses.

Trichoxanthin

This name was proposed by Lillie (1957b) for the yellow granular pigment occurring in the hair medulla of the paler varieties of guinea-pig. Its histochemical characteristics strongly resemble those of the melanins and Lillie suggests that, like these pigments, trichoxanthin contains quinhydrone groupings.

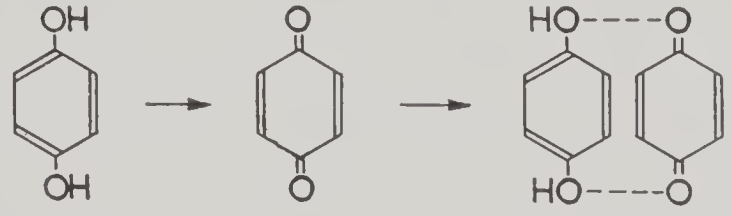
Arenichrome

A green granular pigment in the epithelial cells of *Arenicola marina* L. was named arenichrome by Lignac (1945) who described its histological and histochemical characteristics. This pigment had already been noted by Fauvel (1899) under the title "lipochrome jaune". This author described the solubility of the pigment in alcohol, its preservation by alkali, and its conversion by the influence of light, especially at acid pH levels, into a brownish-black insoluble pigment. Lignac, agreeing with these findings, regarded arenichrome as a coloured premelanin, capable of acting as a "mesocatalyst" or respiratory carrier pigment. Later studies by Van Duijn (1952) resulted in the extraction and crystallization of arenichrome as its

potassium salt ($C_{21}H_{15}S_2K_3O_{14}$). The presence of sulphur in the form of strongly acid groups, presumably SO_3H, provides a point of similarity to the melanins. The absence of nitrogen, however, must place arenichrome in an entirely different category. Further histochemical studies on arenichrome have not been carried out.

Adrenochrome and the Chromaffin Reaction

Older Views on the Chromaffin Reaction. The observation that certain cells in the adrenal medulla contained dark brown material in granular form, after fixation with dichromate or chromic acid, was made by Henle in 1865. The chromaffin reaction, as it has long been called, was attributed to adrenalin by Meulon in 1905. Verne (1923) showed that not only adrenalin, but also other aromatic compounds containing two OH or NH_2 groups *ortho* or *para* to one another, would give the brown colour on reaction with dichromate. The experiments of Ogata and Ogata (1923), who demonstrated the presence of chromium in the chromaffin granules, seemed to indicate that the reaction depended on the reduction of dichromate to a yellow or brown compound. Gérard, Cordier and Lison (1930) proved, however, that it was due to an oxidation of the reacting substance by the dichromate. They were able to obtain the typical result by fixation in a fluid containing the mild oxidizing agent potassium iodate. Lison (1936) pointed out that these findings increased the specificity of the reaction which, on the former hypothesis, could be due to any reducing substance. He considered that the reacting substance was converted into a quinone which then coupled with a molecule of the original substance to give a deeply coloured quinhydrone.



Colourless Pale yellow Brown

From this reasoning Lison put forward four criteria of chromaffinity : (1) All substances which belong to the series of aromatic photographic developers are chromaffin. (2) The reagents giving the chromaffin reaction are oxidants which do not act too energetically. (3) The final coloured product is a quinhydrone. (4) Decolorization of this quinhydrone is produced by strong oxidation.

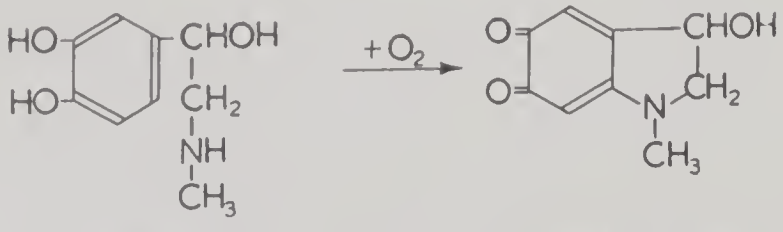
Lison stated that fixatives containing $HgCl_2$ as well as dichromate gave only inconstant results and that those containing chromic acid generally gave negative results. He recommended the use of formalin containing potassium iodate, since this avoided the pseudo-chromaffin

reaction given by substances capable of absorbing chromium salts. Other authors recommend a simple formalin-dichromate solution. Lillie (1948) could not obtain the chromaffin reactions with Lison's fixative, and he stated that as little as one hour's preliminary treatment with formalin would prevent production of the reaction by dichromate.

It is sometimes helpful, though never diagnostic, to stain the chromaffin granules, subsequent to fixation with dichromate, by some method like that of Wiesel (given by Roulet, 1948). This depends on staining with aniline blue or toluidine blue followed by nuclear staining with safranin ; chromaffin cells appear green, other cells blue. Alternatively, modifications of Schmorl's Giemsa method can be employed to give a similar final effect. Sevki's (1934) modification of Schmorl's method (Appendix 23, p. 921) was successfully employed by Blacklock *et al.* (1947) to demonstrate chromaffin granules in cases of phæochromocytoma. This method, originally designed for use with non-chromated tissues, gives a pink colour with chromaffin cells under such conditions. In the case of chromated tissues, however, the colour is green.

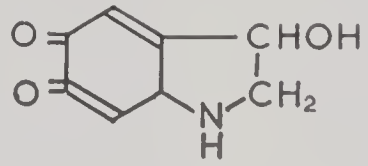
Lillie (1948) mentioned, but did not explain, the fact that chromaffin granules stain red by the PAS technique. I could not produce a positive PAS reaction in the chromaffin granules of either human or dog adrenals. The cytoplasm of the chromaffin cells, however, stains deeply by this method when an aqueous solution of HIO_4 is employed as the oxidizing agent. It is theoretically not impossible for noradrenochrome (see formula below) to react with Schiff's reagent since it contains two carbonyl groups, one of which at least is reactive. Adrenochrome itself should not react since it is a tertiary amine. The effect of periodic acid on tissue chromaffin, which is normally Schiff-negative, might be to release one or both of the carbonyl groups for combination with Schiff's reagent, or perhaps to produce an aldehyde group from some other part of the molecule.

Modern Views on the Adrenochromes. More modern work on the pigments derived from the oxidation of adrenalin has led to modifications of the views put forward by Lison although it has not significantly altered the histochemical significance of the reaction. Green and Richter (1937) produced a deep red compound (adrenochrome) by the oxidation of adrenalin with catechol oxidase. They advanced the following equation for this reaction.



Adrenalin Adrenochrome

Reduction of the product, with H_2S or SO_2, gave a colourless leuco-adrenochrome which reacted with ferric chloride to give the typical green colour of a catechol. A similar mechanism is presumed to operate in the case of noradrenalin, to produce the pigment noradrenochrome whose formula is shown below:



Noradrenochrome

Richter and Blaschko (1937) obtained a red iodo-adrenochrome by treating adrenalin with iodate in acid solution. This finding suggests that the success of Lison's iodate method depends on the presence of sufficient acidity in the formalin which is employed. Using iodate-formalin solutions I found that "chromaffin" was produced between pH 5·0 and 6·8 but that the amount decreased if the iodate-containing formalin was more acid or more alkaline than this. Lillie's failure to produce chromaffin granules with the iodate-formalin fixative was therefore perhaps explicable on the basis of insufficient acidity, since the formalin he used was buffered. The reaction product of Lison's method was presumably iodo, rather than simple, adrenochrome. It reacts essentially in the same way as the substance produced by dichromate oxidation, but is less strongly reducing towards ferricyanide and silver solutions.

Distinction between Adrenalin and Noradrenalin. In a series of papers (1954a, 1954b. 1955) Hillarp and Hökfelt described a method for the distinction of noradrenalin from adrenalin and its application to the adrenal medulla in a number of different species. These authors found that when oxidation was carried out with potassium iodate at pH 5–6 noradrenalin was converted into a dark pigment within a few minutes while adrenalin, on the other hand, required about 24 hours. Oxidation with chromate or dichromate rapidly produces brown insoluble pigments from both adrenalin and noradrenalin and the classical chromaffin reaction thus demonstrates both these substances.

The original medium used by Hillarp and Hökfelt was a buffered solution containing 2·5% iodate but this was found unsatisfactory both by the authors and by other workers (e.g. Eränkö, 1955a). A 10% solution of iodate (unbuffered) was therefore substituted. Only thin tissue slices can be used for the method, which is given in full in Appendix 23, p. 921. Frozen sections or freeze-dried sections are quite useless. The localization of noradrenalin afforded by this method is histologically satisfactory. Within the cell, however, diffusion takes

place so that the nucleus is stained brown. Its application to phæochromocytomas was described by Ghislandi *et al.* (1955).

The Fluorescence Method. It was noted by Eränkö (1951, 1952) that after fixation in formalin some of the cells of the adrenal medulla showed a strong greenish fluorescence while the rest were only feebly fluorescent. By means of a microdissection technique and chromatography Eränkö (1954) was able to confirm that the fluorescing islets contained noradrenalin and later (1955b and c) he showed that there was an excellent correlation between noradrenalin content and fluorescence in seven species of mammals.

In vitro experiments showed that noradrenalin and formalin formed an insoluble fluorescent compound but that adrenalin did not react in this way. Comparative studies made by Eränkö (1955a, 1956) of the fluorescent and iodate methods for the demonstration of noradrenalin led to the conclusion that the two reactions gave positive results in the same types of medullary cell and that either method might therefore be employed with confidence. In practice, the iodate method is easier but cannot be used on fresh frozen sections. If fluorescence microscopy is available I consider the fluorescent method, applied to cold microtome sections, to be the method of choice. Details are given in Appendix 23, p. 922, and the two methods are illustrated in Figs. 202 and 203.

Other Methods for Adrenalin. Coujard and Coujard-Champy (1942) described the use of an osmium tetroxide-potassium iodide mixture for demonstrating adrenalin in sympathetic nerves and adrenal medulla. This method which was also used by Champy and Hatem (1955) has been criticized by Lison (1953) as unspecific. He states that although the mixture is reduced particularly strongly by phenols it reacts in tissue sections with many other types of compound.

Relationship of Chromaffin Granules to Mitochondria. Histologists have long supposed that adrenalin in the chromaffin cells was present in the form of granules. Blaschko and his associates (Blaschko and Welch, 1953; Blaschko, Hagen and Welch, 1955) have made a particular study of this problem and they find that adrenalin is certainly contained in the large granule fraction of sucrose homogenates. From these granules it is easily released by acetolysis, high temperatures and by electrolytes. Feldberg (1940) and Hillarp and Nilson (1954) both described the release of adrenalin after treatment of the granules with lysolecithin and the latter authors described the liberation of both adrenalin and noradrenalin by hypotonic solutions of electrolytes and non-electrolytes and by lecithinase A. All these observations suggest that the adrenalin-containing granules possess some kind of membrane and Hillarp *et al.* (1954) consider that they are probably mitochondria. Blaschko, however, believes that this hypothesis

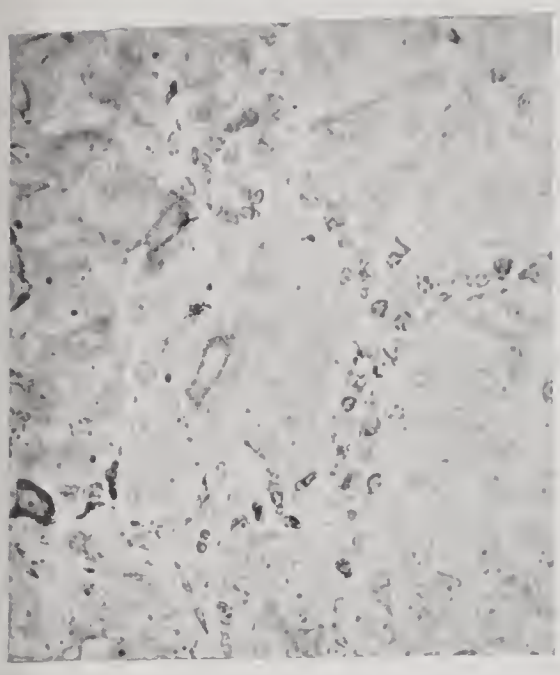


FIG. 200. Benign nævus-cell melanoma. Melanin granules appear black. Masson-Fontana stain. × 370.

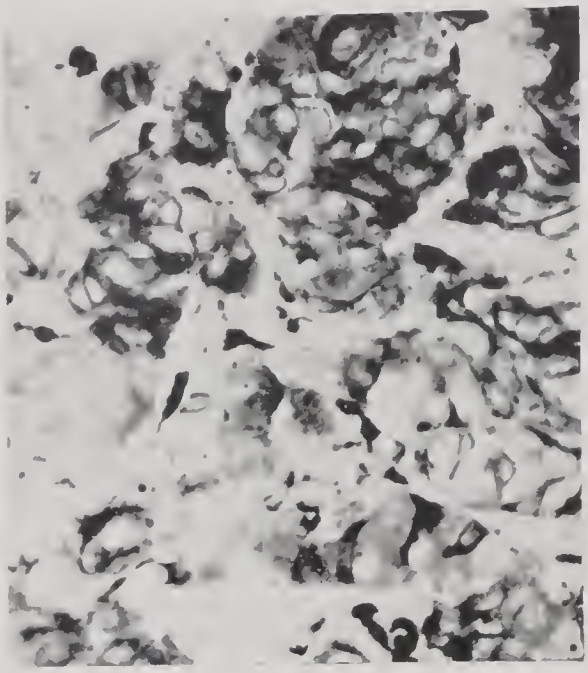


FIG. 201. For comparison with Fig. 200. A similar field in a similar section stained by Schmorl's method. The degree of staining is much greater. × 370.

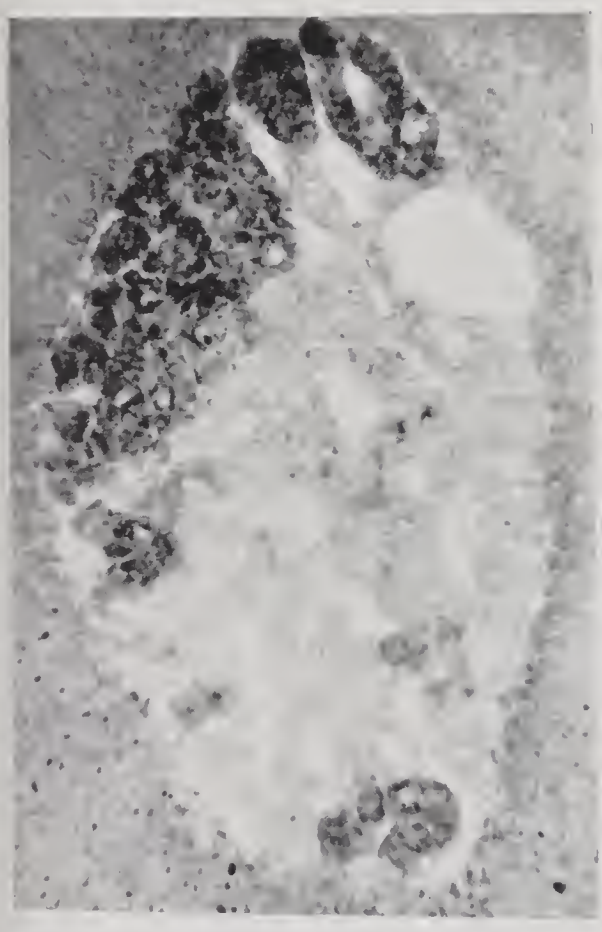


FIG. 202. Hamster adrenal gland. Iodate reaction shows brown pigment from noradrenalin in islets in the medulla. × 60.

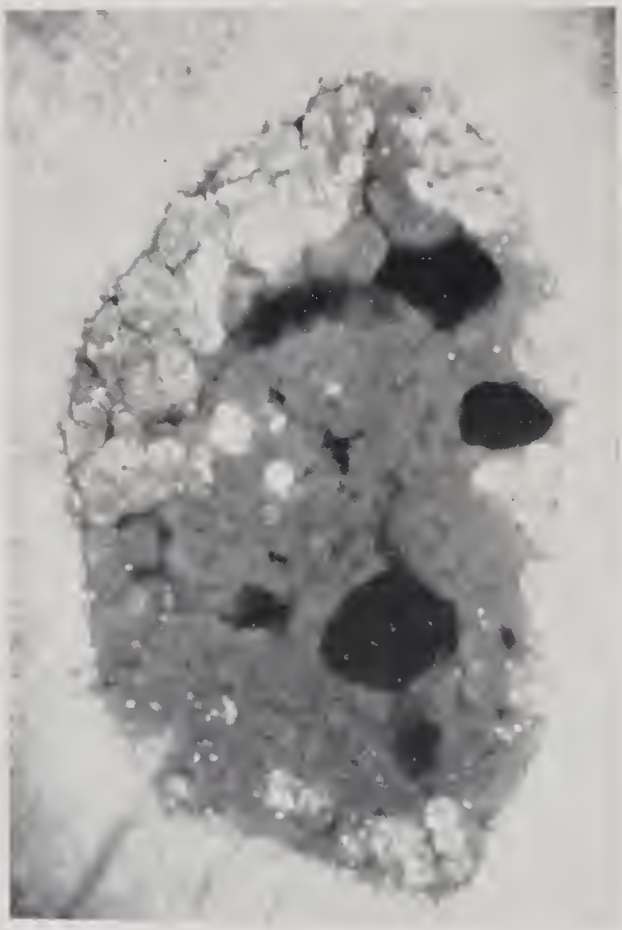


FIG. 203. As Fig. 202 but contralateral adrenal. Formol calcium—induced fluorescence in peripherally situated islets in the adrenal medulla. × 60.

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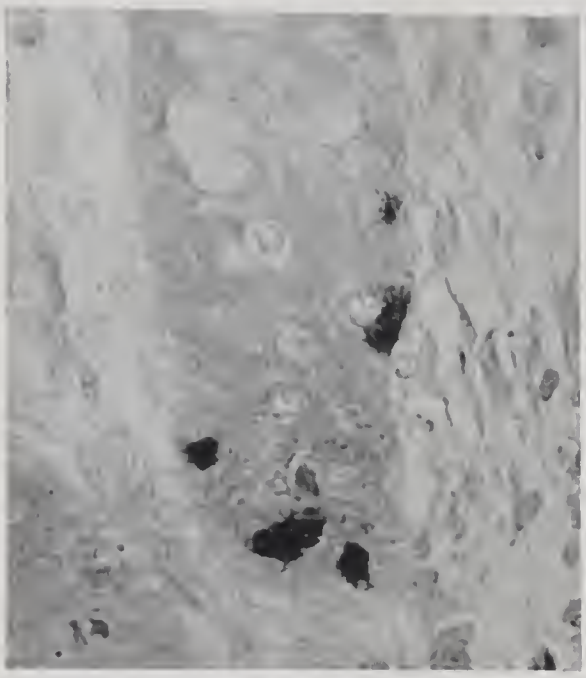


FIG. 204. Paraffin section (5·5 μ) of human small intestine. Argentaffin granules are stained black. Gibbs' reaction, carmalum. × 460.

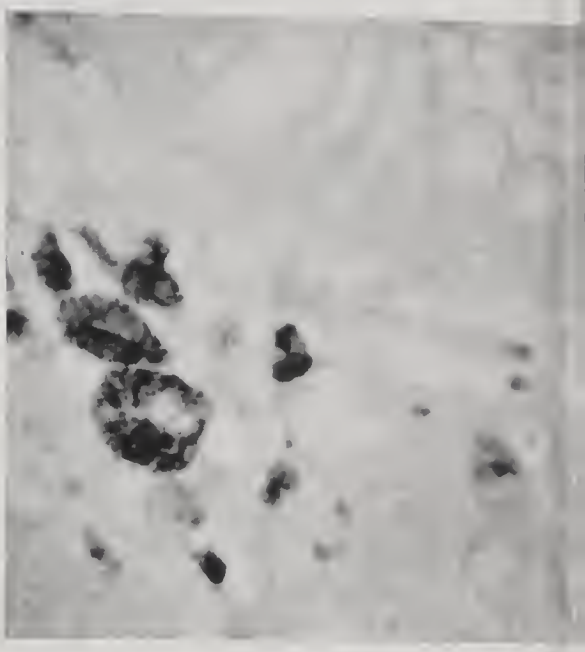


FIG. 205. Human appendix (paraffin sectio Showing, in the lamina propria (*left*) strong staining " pseudomelanin " granules and a (*right*) argentaffin granules. Schmorl's action. × 520.

FIG. 206. *Bombinator pachypus*. Skin. Formalin-fixed. Shows (*left* and *right*) silvery-white fluorescent granules and (*centre*) yellow fluorescent granules in cutaneous glands. × 45.

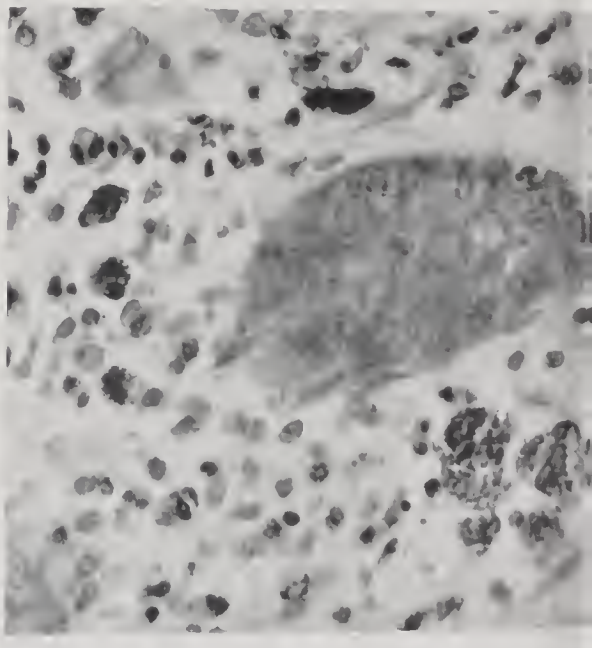


FIG. 208. As Fig. 205. " Pseudomelanin granules in macrophages in the lamin propria showing various degrees of positiv staining. Periodic acid-Schiff, hæmalu × 370.

cannot yet be taken as proven. Both the fluorescence and iodate methods should obviously be reapplied to sections of adrenal gland cut on the cold microtome and maintained in a cold "protective" environment while the respective reactions take place.

Tryptophan Pigments and the Indole Reaction

Little attention has been given to the histochemistry of the red pigments derived from tryptophan, which can be extracted from the urine and which may also occur in the tissues, if only in the form of intermediates. They are better considered at this point than in Chapter V, into which they do not fit readily because they are not demonstrable by the indole reaction.

The tryptophan pigments are of two classes, the indigoids, formed by the oxidation of free tryptophan, and the Hopkins-Cole pigments, which are formed from free or peptide-linked tryptophan by condensation with an aldehyde and subsequent oxidation. Fearon and Boggart (1950) obtained a fluorescent red pigment from tryptophan by oxidation with iodate in acid solution, which they called tryptochrome. This was found to be a very unreactive substance, insoluble in water and dilute alkalis but soluble in organic solvents and especially in chloroform. If a pigment such as tryptochrome was formed in the body it might be expected to be stored in solution in lipids as are the fat-soluble carotenoid pigments. No reactions have been described by which tryptochrome can be demonstrated histochemically although it has a characteristic fluorescence spectrum.

Enterochromaffin (Kultschitsky) Cell Granules

In the first edition of this book the Kultschitsky cells were described as argentaffin cells. This title has been changed in favour of the term enterochromaffin (E.C.) cells largely in acknowledgement of the great contributions towards an understanding of their functions made by Erspamer, Vialli, and others of the Italian school. Most published work on the E.C. granules refers to the characteristic strongly acidophil granule produced by formalin fixation. To say that this must be regarded as an artifact does not mean that granules cannot be preserved in E.C. cells except by formalin. As recently pointed out by Christie (1955b) such granules were demonstrated by Nussbaum (1879) and by Grutzner and Menzel (1879) after fixation with osmium tetroxide. Christie himself observed granules after fixation with dichromate. It is necessary, though pedantic, to point out that the OsO_4 granules are not argentaffin and that neither they nor the chromate-fixed granules give many of the characteristic histochemical reactions described below.

Major Histochemical Tests. Five positive histochemical tests give

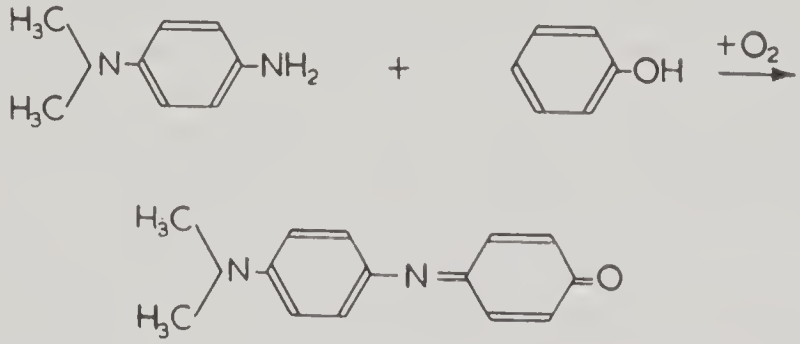
insight into the chemical nature of the granules. These are the *argentaffin*, *chromaffin*, *diazonium*, *indophenol* and *Gibbs'* reactions. A few subsidiary tests and characteristics are also of sufficient interest to be mentioned here. Their property of reducing ammoniacal silver solutions to metallic silver (argentaffin reaction) is most commonly employed by histologists for demonstration of the granules. The most popular techniques are those of Masson-Hamperl, Masson-Fontana and the newer Gomori hexamine-silver method ; the last two are given in Appendix 23, p. 919. The argentaffin reaction is of low chemical specificity, as pointed out by Lison, who considered that it was given particularly strongly by dihydric and polyhydric phenols, aminophenols and polyamines, and less strongly by many other substances, so that for practical purposes in histochemistry a positive reaction suggests the presence of phenols. Cordier (1927), Hamperl (1932), Lison (1936) and Gomori (1948) have all strongly stressed the fundamental difference between the argentaffin reaction and silver impregnation methods, in that the former is brought about by the reducing capacity of the tissue component itself and the latter by the addition of an extraneous reducer. It is still necessary to stress this point although it now appears to be entirely justifiable to diagnose argentaffin cell tumours (carcinoids) on the basis of granules demonstrated solely by impregnation techniques employing a reducer. The position is not as simple as it might be because, if tissues containing argentaffin cells are fixed in alcoholic fixatives, so that the specific granules are absent, the cells which contained the precursor substance can still be demonstrated by a reducing impregnation technique. That is to say, the cells are still argyrophil though they are no longer argentaffin. If the argyrophil method is applied to formalin-fixed tissue, intestine for instance, the morphology and position of the resulting argyrophil cells closely resembles that of the argentaffin cells. Numerically, however, they exceed the latter by a considerable amount. Hamperl (1951, 1952) believed that the argyrophil but non-argentaffin cells were precursors of the argyrophil *and* argentaffin (true argentaffin) cells. Sharples (1945a and b) observed that the Bodian (reducing) silver technique often demonstrated large numbers of argyrophil cells in the deeper layers of the stomach mucosa, and Gomori (1948) stated that the majority of these cells, though scarcely recognizable by the usual (non-reducing) argentaffin techniques, were well shown by his hexamine-silver method. The same cells were demonstrated by the other specific reactions mentioned in this chapter but in shades so pale that they were only recognizable with difficulty. This observation seems to accord well with Hamperl's hypothesis but Clara (1957) believes that only a proportion of the argyrophil basal granulated cells can be regarded as precursors of the E.C. cells.

Certainly anyone who is investigating the Kultschitsky cells, from either the morphological or the histochemical point of view, should not overlook the value of the argyrophil reactions in demonstrating them.

Returning to the histochemistry of the granules, it is not necessary to mention the *chromaffin reaction* in this context, other than briefly. It was considered by Lison to be a specific reaction solely exhibited by the dihydric and polyhydric phenols, aminophenols and polyamines. It has not been used for the diagnosis of carcinoid tumours and is seldom employed for the demonstration of the specific granules in intestinal cells. Nevertheless a positive chromaffin reaction can be obtained either with iodate or chromate after formalin fixation, since the specific substance of the E.C. cells is preserved by the latter.

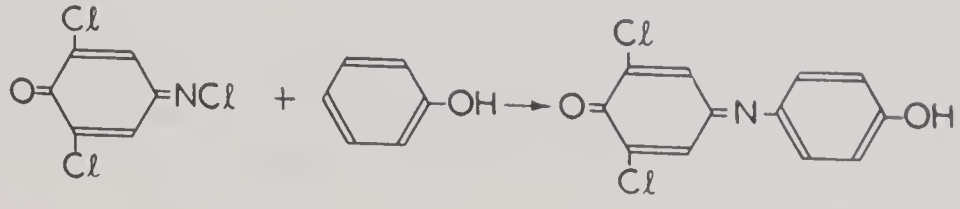
The *diazonium reaction* demonstrates the granules particularly clearly (Plate VIIIc) and the resulting colour varies with the particular *diazonium* salt which is employed. Technical details of the method, and considerations of the various salts which can be used, appear in Appendix 23, p. 922. Lison considered that the diazonium reaction, in alkaline solution, was given by all phenolic substances on condition that the hydroxyl was unsubstituted and that at least one of the positions *ortho* and *para* to the hydroxyl group was also free.

The *indophenol reaction* (Lison, 1931) depends on the production of a blue or green indophenol dye when a phenol combines in the presence of an oxidizing agent with dimethyl*para*phenylenediamine, as shown in the equation below.



According to the author, the technique was difficult in application and, in practice, the colour developed is too pale to be of much use in diagnosis.

The fifth and last of those main reactions which we have been considering is Gibb's reaction (Gibbs, 1926). This is based on the formation of an indophenol dye when 2 : 6-dichloroquinonechloroimine reacts with a phenol in alkaline solution.



The final colour depends on the phenol with which the quinoneimine is coupled. With phenol itself, or α- and β-naphthol, it is blue, and with resoreinol reddish-purple. Coupling does not occur with catechol or with any of the *para*-substituted phenols. The use of this reaction in the particular instance of the argentaffin granules was first described by Gomori (1948) and the black or brownish-black colour developed in tissue sections is very satisfactory. Fig. 204 illustrates the results of its application to material from the human intestine.

Other Histochemical Tests. Four lesser characteristics of the E.C. granules may well be described at this point. The first is the *Vulpian reaction* (Vulpian, 1856) which is given, according to Lison, by all *ortho*diphenols (catechols). It depends on the development of a green colour when a very weak solution of ferric chloride is applied to tissues containing reactive phenols. Vulpian himself used the reaction to demonstrate the substance subsequently identified as adrenalin in the adrenal medulla, and it was observed by Jonnard (1934) to give a weak reaction with the argentaffin granules.

In a large proportion of the Kultschitsky cells of the human intestine the granules have sufficient reducing capacity to give a strong blue colour with *Schmorl's ferric-ferricyanide reaction*. This reaction is of no use in elucidating the exact chemical nature of the granules, since it indicates only the presence of a substance capable of reducing ferricyanide to ferrocyanide. The property is shared, *inter alia*, by the melanins, the lipofuscins (below), sulphydryl groups (Chapter V, p. 108), and probably by aldehydes such as plasmal (Chapter XII). It is necessary to remember these facts when attempting the differential diagnosis of obscure pigment granules having reducing properties towards a number of reagents. Fig. 205 shows argentaffin granules in the human appendix stained by Schmorl's reaction.

The fact that the E.C. granules give a positive acid hæmatein reaction was recorded by Christie (1954) who showed also that the reaction was negative after pyridine extraction. In my hands, using duodenum from guinea-pig, rat and man, the reaction is always a weak one but definitely positive in a proportion of the E.C. cells in all three species. In a number of carcinoid tumours only an occasional positive cell was recorded. The interpretation of a positive acid hæmatein reaction (with negative pyridine-extracted control) is not usually difficult and the presence of phospholipid can be confirmed by a number of alternative reactions. I have consistently failed to demonstrate the E.C. granules by means of any other phospholipid reaction. Moreover, it can be shown that the condensation product of 5-hydroxytryptamine (5-HT) and formaldehyde, after postchroming as in Baker's method, can be stained with acid hæmatein. This condensation product, whose use was suggested by the work of

Erspamer and his associates referred to below (p. 649), and the phenolic material in the fixed E.C. cells, are both soluble in hot pyridine so that in this case the control reaction has no differential significance. It is reasonable to suppose that the water-soluble phenol present in the E.C. cells occurs in bound form and one of the most likely forms of binding would be with lipid or phospholipid. Christie's interesting hypothesis cannot be sustained, however, unless some corroborative evidence of the presence of phospholipid in the granules can be produced.

The last of the four lesser characteristics referred to is the yellow *autofluorescence* shown by the granules when they are exposed to ultra-violet light. This was described initially by Erös (1932). Jacobson (1939) measured the fluorescence spectra and ultra-violet absorption spectra of sections of a human carcinoid tumour. The fluorescence spectra were shown to have two bands, one with a peak at 6100Å, the other at 5500Å. The U.V. absorption maximum was at 2700Å. In a second paper (1946) this author claimed that the fluorescence spectrum of an ethylene glycol monoethyl ether extract of a carcinoid tumour was almost the same as that given by a solution of xanthopterin in this solvent. From his spectrographic results and those of certain histochemical tests (notably the murexide test) Jacobson suggested that the granules contained a pteridine compound. Xanthopterin and leucopterin, the yellow and white pigments of insects, are the two most important members of this class. Gomori (1948), however, tested 2-amino-4-hydroxypteridine-6-carboxylic acid by the five major reactions detailed above and in no case did he obtain a positive result. Personally, I have not succeeded in demonstrating a positive murexide test in the granules from mammalian sources. Jacobson's views have not been substantiated and this suggests that the results of fluorescence spectrography cannot always be taken as reliable unless they are supported by independent histochemical and, if possible, chromatographic evidence.

The fluorescence of freeze-dried rat duodenal E.C. cells was obtained by Barter and Pearse (1953, 1955) only after exposure of the sections to formalin vapour. Substances suggested by various authors as possible precursors of the fluorescent compound were tested *in vitro*. Of these only 5-HT was found to be non-fluorescent before and fluorescent after treatment with formaldehyde. With a better source of UVL I have since found that unfixed 5-HT has a weak blue fluorescence easily distinguished from the bright yellow fluorescence of the complex. On account of their findings Barter and Pearse postulated that a harmaline derivative or, more probably, a fully conjugated β-carboline (Jepson and Stevens, 1953) was the type of substance actually present in formalin-fixed E.C. cells and that this was therefore responsible for the classical reactions of the granules.

The ninhydrin reaction. A specific test for tryptamines unsubstituted at position 2 and either of the nitrogen atoms was described by Jepson and Stevens (1953). A bright blue-green fluorescence is given by 5-hydroxytryptamine, on filter paper, after treatment with 0·2% ninhydrin in acetone containing 10% v/v acetic acid, drying and heating to 100° for 2–3 minutes. This reaction, applied to freeze-dried sections of duodenum, gives negative results. These might be due to quenching effects from the blue ninhydrin-amino-acid reaction product but more probably they are due to the fact that adsorption of the compound on filter paper is necessary for a positive reaction to occur.

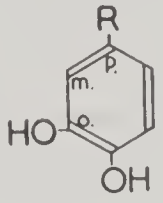
There are some considerable objections to the hypothesis of Barter and Pearse. The first of these is that the *in vitro* condensation of 5-HT with HCHO is slow and markedly pH dependent. In the tissues it appears to be more rapid and relatively independent of pH. I believe this discrepancy will ultimately be resolved when the nature of the structure responsible for binding the product within the E.C. cell is known. A second objection is raised by the work of Christie (1955a). This author has stated that in unfixed sections of freeze-dried guinea-pig duodenum the E.C. cells fluoresce brightly yellow when irradiated by UVL at 2750Å. The effect of formalin is to diminish the colour and to change it in the direction of orange. I have repeatedly attempted to reproduce these findings in the guinea-pig but entirely without success. In the rat, and in the hamster, there is no doubt that non-fluorescent E.C. cells are made fluorescent by formaldehyde but this effect does not seem to occur in the guinea-pig. The activation maximum for 5-HT is 2950Å (Bowman *et al.*, 1955) and its fluorescence maximum is 3300Å except in strongly acid solution (Udenfriend *et al.*, 1955) where 5-HT and other 5-hydroxy-indoles fluoresce at 5500Å. If, therefore, a yellow fluorescent compound is present in unfixed E.C. cells it is not 5-HT.

A third objection to the hypothesis of Barter and Pearse comes from further studies by Jacobson (1958) who claims to have shown that the fluorescence of the 5-HT-formaldehyde compound is much weaker than that of the material extracted from a formalin-fixed carcinoid and that it has a slightly lower emission maximum. I believe that the compound selected for comparison by Jacobson was one of the soluble yellow precursors of the β-carboline, which itself is insoluble in nearly all organic solvents. These indeed fluoresce weakly by comparison with the final product.

Neither the identity nor the non-identity of the synthetic and natural compounds can yet be considered proven.

The Identity of the Reacting Substances. Cordier and Lison (1930) made a thorough study of the nature of the argentaffin granules,

using the first four major reactions detailed above and also Vulpian's reaction. From the diazo and indoreactions they concluded the presence of a phenolic group and, from the stability to alkali of the colour developed in the former reaction, that coupling must have occurred in the *ortho* position. This meant that the *para* position was not free since coupling invariably occurs at that point if it is. From the chromaffin and argentaffin reactions, in addition, they concluded that the granules contained an *ortho* or *para*-polyphenol, and with the aid of the Vulpian reaction the structure was imagined as an *ortho*-diphenol (catechol) of the type

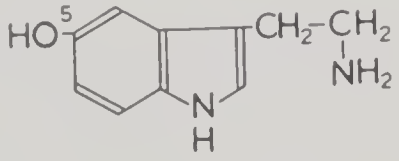


carrying a side-chain in the *para* position. Because the shades of the dyes formed in the diazo reaction were yellowish it was assumed that the side chain was relatively simple.

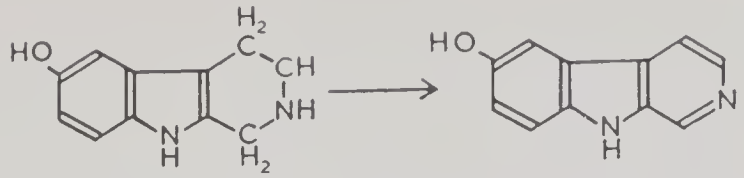
The ideas of Cordier and Lison were challenged by Gomori (1948). This author pointed out that there were three fallacies in their reasoning. First, the positive indophenol reaction is against a *para*-substituted phenol since these dyes do not usually form unless the *para* position is free. Secondly, catechol and its derivatives are far stronger reducing agents than argentaffin granules and, thirdly, there are numerous exceptions to the rule that the colour of the diazo dye obtained from *ortho*-coupled phenols is stable to alkali, while that obtained from *para*-coupled phenols is not. From the result of the Gibbs reaction Gomori excluded the possibility of the granules containing catechols or any *para*-substituted phenol, since with these the reaction is negative. In *in vitro* experiments he showed that catechol or hydroquinone would reduce alkaline silver solutions in a few minutes while resorcinol and phloroglucinol took several hours. Furthermore, short treatment with iodate or dichromate would abolish the silver-reducing power of the first two substances leaving that of the other two, like that of the granules, unchanged. Finally, Gomori showed that the shades produced by the diazo reaction with resorcinol were the same as those given by the granules, and he therefore suggested that these contain a derivative of *meta*diphenol (resorcinol) rather than *ortho*-diphenol (catechol), as previously believed. Following the work of Erspamer and Asero (1952), described in the next paragraph, Gomori (1954) retested the above-mentioned compounds, and also 5-HT, with various diazonium salts. He considered that the colours given

by the E.C. granules resembled those given by resorcinol more closely than those given by 5-HT. The resorcinol hypothesis of Gomori was supported, to some extent, by the results obtained by Lillie, Burtner and Henson (1953) with their diazo-safranin method. It was opposed by Barter and Pearse (1953, 1955) and by the result of the alkaline thioindoxyl reaction (Pearse, 1956). This reaction depends on the fact that formalin-fixed E.C. granules, subjected to mild oxidation, will couple with thioindoxyls to give coloured thioindigoid dyes. It was postulated that the oxidation product in the granules is a quinoneimine since these are known to react at alkaline pH levels with thioindoxyls. Resorcinols do not form quinones, nor do they react *in vitro* by the alkaline thioindoxyl method. Details of this method are given in Appendix 23, p. 923, but it is not recommended for routine studies of the E.C. cells. It was nevertheless applied by Vialli and Quaroni (1956a), with some success, to the demonstration of the cells of the enterochromaffin system in *Rana esculenta*, *Discoglossus pictus*, and *Bombinator pachypus*. The latter, as pointed out by Vialli, is histochemically interesting in that its cutaneous glands are of two types. One, containing large granules, fluoresces yellow after formalin fixation and the other, containing small granules, has a brilliant whitish fluorescence. These appearances are shown in Fig. 206, p. 641. According to Vialli and Quaroni both types of granule gave a positive alkaline thioindoxyl reaction.

A very considerable volume of work by Erspamer and his associates culminated in the identification by Erspamer and Asero (1952) of the "hormone of the enterochromaffin cells" as 5-hydroxytryptamine. This they obtained in the form of its picrate from extracts of the salivary glands of *Octopus vulgaris* (which contain chromaffin cells). The formula of 5-hydroxytryptamine is given below:



It is seen to be a derivative of 3(β-aminoethyl)indole (tryptamine), the latter being produced from tryptophan in the body in the same way as tyramine is produced from tyrosine. This hormone the authors called enteramine. The mechanism by which 5-HT combines with formaldehyde is still uncertain. Nevertheless it seems likely that a Mannich reaction takes place with the active hydrogen atom in position 2, to form a cylic compound (I) and this could subsequently oxidize to the fully conjugated β-carboline derivative (II).



I II

This relatively simple theory is complicated by the fact that active hydrogen atoms are present also at positions 4 and 6 in the 5-HT molecule. Opportunity for the occurrence of polymers is thus present. Probably these are actually formed *in vitro* and even perhaps in E.C. cells subjected to treatment with formaldehyde. On the other hand, in the case of the E.C. cells, the reaction of intermediates with protein might well occur.

Erspamer and Boretti (1950) examined acetone extracts of *Octopus* salivary glands by paper chromatography. They separated a number of enteramine-like constituents, three of which gave the same reactions as enteramine in spot tests applied to the paper. Reactions listed as positive were:

(1) *The acid diazo reaction* with *p*-nitroaniline (Appendix 23). This gave a peach-red colour which turned violet on exposure to ammonia.

(2) *The nitro reaction* ($NaNO_2 + HCl$). This gave a yellow to orange colour.

(3) *The chromaffin reaction.* Brown, violet or red colours developed.

(4) *Ammoniacal silver reaction.* Immediate reduction to black silver occurred.

(5) *The indole reaction* (Appendix 5, p. 798). This gave a blue colour, turning violet and then green.

(6) *The Folin-Ciocalteau reaction.*

(7) *The xanthoproteic reaction.*

(8) *The Millon reaction.* This gave an olive-green colour.

(9) *Fluorescence in UVL.*

All these reactions, except the indole, Millon and Folin-Ciocalteau reactions are positive with formalin-fixed mammalian argentaffin granules, and of these three only the negative indole reaction is significant. The failure of the various indole reactions (*see* Chapter V, p. 92) to demonstrate formalin-fixed E.C. granules has been emphasized by Lillie (1956) and by Glenner and Lillie (1957). If the mechanism of HCHO condensation postulated by Barter and Pearse is accepted this would be accounted for by blocking of the free α-CH group of the pyrrole nucleus which is responsible for the indole reactions of 5-HT. Glenner and Lillie find, however, that serotonin (5-HT creatinine sulphate), fixed in formalin vapour for 48 hours, still reacts by the post-coupled benzylidene method. They consider that such a compound cannot be present in fixed E.C. cells. Furthermore, they find

that when treated with formalin *in vitro* tryptophan and 5-HT lose their activity as indoles at proportional rates. This suggests to them that if one compound reacts in formalin-fixed tissues the other should do likewise. The resolution of these arguments depends on further study; some pertinent observations are given below, however, in the section on the applied histochemistry of the E.C. cells.

The Biological Importance of 5-HT in Mammals. It is generally agreed that 5-HT is a substance of great physiological importance although its exact function under normal conditions has yet to be determined. It is known to be formed by hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP), and by the subsequent decarboxylation of this compound (Udenfriend *et al.*, 1953). The enzyme responsible for this second stage is present in most mammalian tissues and, according to Gaddum and Giarman (1956), the amount parallels to some extent their 5-HT content.

Metastasizing tumours of the E.C. cells (carcinoids, argentaffinomas) have been shown by Lembeck (1953, 1954) and by Ratzenhofer and Lembeck (1954) to contain large amounts of 5-HT and patients with these tumours excrete very large quantities of 5-hydroxyindole acetic acid (5-HIAA), the normal breakdown product. The clinical syndrome produced by argentaffinomas was first recognized by Waldenström and his colleagues (Pernow and Waldenström, 1954) and it is notable that no effects due to the action of 5-HT on the nervous system are recorded. This is because 5-HT cannot penetrate the blood brain barrier although 5-HTP does so readily (Udenfriend *et al.*, 1956). In a case of argentaffinoma with renal metastases reported by Smith *et al.* (1957) large amounts of 5-HTP and 5-HT, as well as some 5-HIAA, were excreted in the urine. These findings, together with other evidence, suggested to Dalgleish and Dutton (1957) that 5-HTP is formed at some central locus and converted elsewhere into 5-HT, and that this locus is very possibly the E.C. cell.

If indeed the E.C. cell is the major site of 5-HTP formation we have to reconsider the known histochemical reactions of the cells with this possibility in mind. All the known positive reactions are compatible with the presence of 5-HTP and once again the only significant negative one is the indole reaction, which would be expected to be positive. Although normal E.C. cells are unstained by any of the existing histochemical methods for indoles the cells of argentaffinomas, particularly at the growing edges of the tumour, react fairly strongly. This may be due to protein-bound tryptophan or to protein or lipid-bound 5-HTP. A possible function of the peptidase found in argentaffinomas by Pearse and Pepler (1957), but absent from normal E.C. cells, might be to enable the tumour to use peptide tryptophan as well as the amino-acid for the synthesis of 5-HTP.

Applied Histochemistry of the Enterochromaffin Cells

In order to test both qualitatively and quantitatively the hypothesis that 5-HT is the chromogenic material in the E.C. cells Benditt and Wong (1957) carried out a number of parallel studies with fixed guinea-pig duodenum and with 5-HT in gelatin. They found that the shades produced in the gelatin models with the alkaline diazonium reaction (Fast Garnet GBC) most closely resembled those of the E.C. cells when the concentration of 5-HT was 5 mg./ml. A reaction was clearly apparent at 1·25 mg./ml. and barely apparent at 0·63 mg./ml. The indole reaction, positive in unfixed gelatin blocks, was uniformly negative after treatment with formaldehyde even when the concentration of 5-HT was 20 mg./ml. These findings are opposed to those of Lillie (1956), which have been referred to on p. 648. Benditt and Wong concluded that 5-HT was certainly the material contained in the E.C. cells and by combining assay methods with cell volume estimations they calculated that the intracellular concentration was 5·4 mg./ml. (minimum value). The lower limit of visibility for 5-HT, stained by histochemical procedures, was found to be about 1 mg./ml. The fact that mast cells, which contain 0·5 mg./ml. cannot be demonstrated by histochemical methods for 5-HT is thus shown to be due to lack of sensitivity of the reactions. Rabbit platelets, which were found to contain between 0·5 and 2·5 mg./ml. of 5-HT gave pale but positive argentaffin and diazo reactions in formalin vapour-fixed smears.

Information from Reserpine Experiments. Reserpine is a crystalline alkaloid extracted from plants of the genus Rauwolfia (*Apocynaceæ*). Moderate doses of this drug were shown by Pletscher *et al.* (1955, 1956) to diminish the level of 5-HT in blood, brain and intestine of rabbits. This observation was naturally followed by studies on the effect of reserpine on the E.C. and argyrophil cells of various species. Vialli and Quaroni (1956b) observed in the cock that 10 mg. per kilo of reserpine would produce in 24 hours a significant decrease of E.C. cells with a corresponding increase in argyrophil cells so that the total number of cells of the two types remained substantially the same in control and treated birds. In guinea-pigs and rabbits Zbinden *et al.* (1957) found that disappearance of E.C. cells from the duodenum was proportional to the dose of reserpine administered. The argyrophil cells were observed to be present in undiminished numbers. Benditt and Wong made very similar observations in the case of reserpine-treated guinea-pigs.

The facts given above clearly indicate a close relationship between the number of E.C. cells in the intestine and the 5-HT levels estimated by assay. They do not prove conclusively that 5-HT is the chromo-

genic substance of the E.C. cells and the possibility remains that it is a precursor substance, bound to protein or lipid. Objections have been made to these hypotheses on the ground that in some animals the 5-HT content of parts of the intestine can be more closely related to the number of argyrophil cells. This alternative hypothesis overlooks the fact that the limiting concentration of 5-HT (or 5-HTP) per unit volume which is recognizable histochemically with present techniques is high and that 5-HT may be, and is, present in large amounts in histochemically negative tissues because of its diffuse localization. There is nothing to suggest its production in these regions except by the mechanism postulated by Dalgleish and Dutton, that is to say by decarboxylation of 5-HTP.

Conclusions. Provided that morphologically recognizable granules are present in the cells of a presumed carcinoid tumour the standard diagnostic reactions (alkaline and acid diazonium; Gibbs test) should be positive. The finest granules are found, in man, in inactive portions of intestine, such as those rendered immobile by Krohn's disease, in well differentiated portions of "intestine" in mediastinal and ovarian teratomata and in many examples of the so-called pseudomucinous cystadenoma of the ovary. The latter should probably be regarded as teratomata. In such regions as these there is no stimulus for discharge of the precursor material, which therefore accumulates. In many carcinoid tumours, however, the material in the cells is excreted as fast as it is formed and no granules can be seen in formalin-fixed sections. It is usually possible even in such inactive cases as this to find a few relatively inactive groups of cells in which positive diagnostic reactions may be obtained. The relationship of tumours composed of argyrophil cells to diazo-positive carcinoids containing E.C. cells remains undetermined.

The Hæm Group

Hæmoglobin

This histochemistry of the hæmoglobins is divisible into two parts, that of the globins, already dealt with in Chapter V, and that of the hæmatins, which we must consider here. Whether the globin is present or not makes little difference in the tests which we apply for the hæmatin part of the molecule. As Lison has stated, the histological demonstration of hæmoglobin is not easy and distinction between the various types can only be made spectroscopically or by absorption spectrography. The former method is satisfactory when applied to pigments *in vitro* but not when applied to tissue sections.

As far as chemical methods are concerned, with one dubious exception, we have only a choice of modifications of the peroxidase

reaction, and these are equally positive with the native globins (reduced and oxy-hæmoglobin, met- and sulphæmoglobin), denatured globins (hæmochromogen and parahæmatin), and with the globin-free compounds (hæmatin, hæm and hæmin). Peroxidase reactions are negative with the porphyrins, hæmatoidin and hæmosiderin. The most commonly employed methods are those using benzidine and fixed rather than fresh material. Lison (1936, p. 249) used a formalin-lead acetate mixture in order to convert hæmoglobin into hæmatin, but he also recommended the method of Slonimski and Lapinski which involved fixation in ferricyanide-formalin for 24 hours. This converted the hæmoglobin into methæmoglobin. It is now more usual to employ formalin fixation and some modification of the benzidine-nitroprusside methods of Lepehne (1919) and Pickworth (1934). As an alternative to these procedures one may use the leuco-Patent Blue method of Lison (1938) or modifications such as those of Dunn (1946) and Dunn and Thompson (1946) using the original leuco-dye or leuco-Cyanol (see Chapter XVIII, p. 521, and Appendix 18, p. 904). It is possible to stain hæmoglobin by means of Perls' reaction if sections are treated for a short period with 10 per cent. H_2O_2 before application of the iron reagent. This method gives only a bluish-green tinge to the red cells and it has no important practical application. Similar treatment with H_2O_2 will sometimes reveal hæmosiderin iron which has not proved reactive when treated with the HCl of Perls' reagent alone.

With all these methods the primary objective has usually been the clear demonstration of red blood cells by virtue of their hæmoglobin content. For this purpose there is really very little to choose between the peroxidase techniques and simpler staining methods for hæmoglobin such as Lendrum's (1949) kiton-red—almond green. Methods revealing the lipoprotein envelope of the red cell give equally good results (Sudan black B or Baker's (1946) acid hæmatein method, both after proper fixation). Hæmoglobin outside red cells is much less easily demonstrated since none of the available reactions possess a sufficiently high degree of specificity. It is possible to distinguish between tissue peroxidases and hæmoglobin and its derivatives, since the former are heat labile and destroyed by warming the slides at 90° (Lison recommended 180°) for 10–20 minutes, while hæmoglobin is not. There are, however, certain other thermostable tissue peroxidases which appear during the process of autolysis. Benzidine methods give a positive reaction with these, in cell nuclei for instance, but Lison stated that his zinc-leuco method did not do so. In practice these objections are not found to carry much weight and the development of a "pseudoperoxidase" reaction can be taken to indicate the presence of hæmoglobin or hæmochromogen. Gomori (1951) considered the zinc-leuco methods to be superior to all others for this purpose

but noted (1952) that only intact hæmoglobin, and not its protein-free derivatives, would stain properly by these methods.

The Porphyrins

These substances, which normally only occur in small quantities in most animals, are considered to be precursors of the hæm part of hæmoglobin. They possess the same tetrapyrrole nucleus as hæm but lack the central atom of iron. Of the large number of possible tetrapyrroles only two types occur in nature, the so-called Series I and Series III porphyrins. Those of Series III are the direct precursors of hæm and those of Series I are regarded as useless by-products of hæmoglobin synthesis. In the fœtus, where this process is vigorous, porphyrins of both series are present in greater amounts than in the adult but, while those of Series III are utilized at once, those of Series I accumulate and are demonstrable in the bone marrow and also in the meconium. In the adult increased amounts of porphyrin (Series I) are excreted in various anæmias and in the congenital or acquired porphyrias they may be present in large amounts in the tissues.

Unfortunately, although the chemistry of the porphyrins is fascinating (see Lemberg and Legge, 1949), their histochemistry is unsatisfactory and disappointing. The only method so far employed for their demonstration makes use of the red and orange fluorescence given by their solutions in ultra-violet light. Lison (1936, p. 255) described this fluorescence as secondary, since in his view it was produced only after treatment of the porphyrins with appropriate solvents. He noted that in alkaline media ultra-violet light rapidly destroyed the specific fluorescence. Since reddish fluorescence is produced by other substances besides the porphyrins (e.g. carotenoids, lipofuscins, oxidized cytochrome), it is necesary to make spectroscopic (or better spectrographic) examination in order to be certain about their presence in the tissues. Spectroscopic analysis has been applied by Sjöstrand (1946) to fluorescent substances in freeze-dried material mounted in inert media, and the freeze-drying technique is particularly suited to such a purpose. Histochemical studies of the porphyrins have been made by Borst and Königsdorfer (1929), in a case of congenital porphyrinuria, by Ellinger (1938, 1940) and Dobriner and Rhoads (1940), and by Grafflin (1942) on the Harderian gland of the albino rat. This last tissue is the classical material for most experimental studies in porphyrin histochemistry. Gillman *et al.* (1945a and b) reported brief investigations of porphyrins present in the liver in cases of pellagra, using a fluorescence technique with fixed frozen sections. They described the fluorescence as intensely scarlet red, localized to hepatic cells and intensified by acids and alkalis. The red fluorescence noted in erythrocytes in cases of anæmia by Seggel (1940)

has been attributed to the presence of porphyrins. With this last case forming the exception to the rule, it should be made clear that the most that can be achieved at present is a particularly poor localization of porphyrins as a diffuse pinkish-red fluorescence in tissues where they are present in sufficient amount. Figge *et al.* (1953) observed that porphyrins had a tendency to accumulate especially in neoplastic, embryonic and traumatized tissues and also in normal lymph nodes. Klüver (1955) found that the white matter of normal brain, exposed to near ultra-violet irradiation, showed fluorescence with a maximum at 6250Å. He was able to confirm that this was due to the presence of coproporphyrin.

A technique by which the porphyrins react *in situ* with a specific, or relatively specific, reagent is badly needed. Porphyrins can form complexes with metallic ions such as Fe^{++} or Cu^{+} but this property cannot be used as the basis of a histochemical test since these ions also become attached to a wide variety of active groups in the tissues (see Chapter IV).

Hæmosiderins

These pigments occur in the form of yellow to brown granules which are normally always intracellular. They contain iron in an easily unmasked condition and have long been considered to be composed of a protein framework combined with ferric iron in the form of the hydroxide, $Fe(OH)_3$. It was shown by Rich (1924) that histiocytes in tissue culture could break down the hæmoglobin of phagocytosed red cells into an iron-containing hæmosiderin and an iron-free hæmatoidin. The time scale of events was established by Muir and Niven (1935) who injected blood subcutaneously into various animals. As early as 24 hours after injection iron-containing pigment was observed in tissue histiocytes and, in rats and mice, hæmatoidin appeared on the 7th day. In rabbits hæmatoidin was not visible at any stage. Strassmann (1944) observed histiocytes containing hæmosiderin 33 hours after intra-tracheal introduction of blood into rabbits. This same author (1949) showed histiocytes with hæmosiderin granules 48–72 hours after experimental brain injury in mice. In man, a similar occurrence has been noted on the third day (Hammes, 1944), and on the fifth day (Baggenstoss *et al.*, 1943) after ventricular puncture. The work of Rich and of Strassmann indicates that, following hæmorrhage, hæmatoidin appears much later in histiocytes than hæmosiderin, probably not until 10–14 days afterwards.

Fresh hæmosiderin is insoluble in alkalis but soluble in strong acid solutions ; after fixation with formalin, however, it is slowly soluble in dilute acids, best in oxalic, then sulphuric, then nitric, formic and hydrochloric acids (Lillie, 1939). Fixatives that contain acids but no

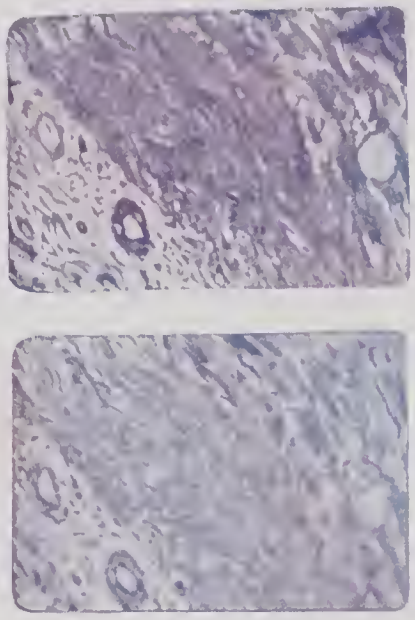
formalin may completely remove hæmosiderin or so alter it that a positive reaction for iron is no longer obtainable. Lillie (1954) gives an excellent description of the hæmosiderins and divides them into three groups, all of which are easily demonstrated by means of Perls' acid ferrocyanide reaction. In his first group he places those hæmosiderins which give a diffuse light blue staining when Perls' reaction is applied. He describes these as showing initially a "diffuse brownish staining of cytoplasm." Very often, however, a diffuse Perls' reaction is given by connective tissues in the vicinity of old as well as recent hæmorrhage, and these are not coloured at all in unstained sections. While the iron may possibly be present here in the form of hæmosiderin, it is equally possible that it may simply be combined with certain acid groups of the protein as is, for instance, the colloidal iron in Hale's method for acid mucopolysaccharides. It is certainly more rapidly removed by treatment with dilute (5 per cent.) oxalic acid than is the iron in hæmosiderin granules. The hæmosiderins in Lillie's second and third groups both occur in granular form but they are distinguished by the fact that one stains slightly with basic dyes (especially thiazines) while the other has no affinity for these.

The constitution of the material remaining in the hæmosiderin granule after removal of its iron has been investigated by Goessner (1953) and by Gedigk and Strauss (1954). Goessner used 5% oxalic acid but Gedigk and Strauss preferred 20% HCl on account of the more rapid and complete extraction of iron which it allows. Both authors found that the carrier substance is a polysaccharide-containing protein giving a strong tetrazonium reaction after benzoylation and being strongly PAS-positive. Gedigk and Strauss studied the development of hæmosiderin after subcutaneous injection of colloidal iron in mice. They found that the basophilia of the carrier substance progressively increased, together with the iron-binding capacity, and that in the later stages (14–84 days after injection) some lipid was also present. The relationship of the iron-binding protein to apoferritin has to be considered, as has the relationship of hæmosiderin to ferritin. The reactions of the latter are dealt with in Chapter XXIV, p. 685.

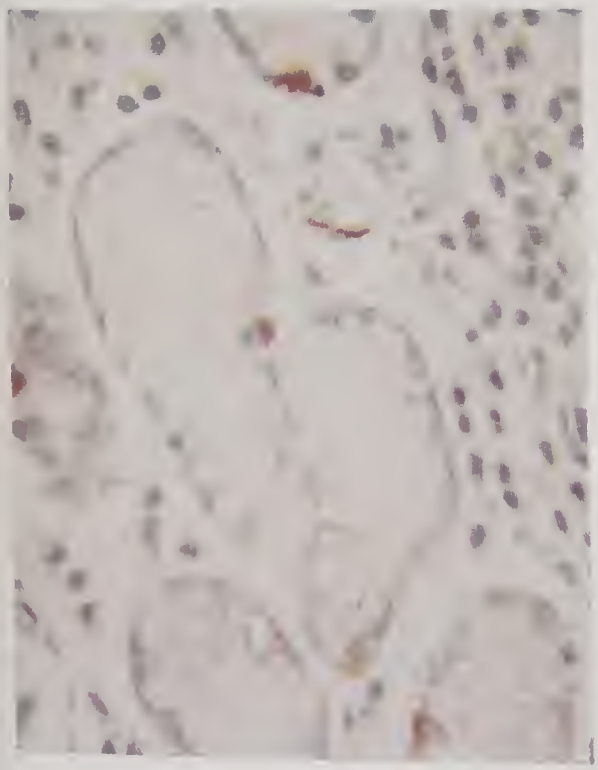
The electron microscope studies of Farrant (1954) and Richter (1957) have thrown much light on the nature of the hæmosiderin granule and the observations of the latter author indicate that ferritin is one of the constituents of hæmosiderin. Richter observes that hæmosiderin is formed within intracellular organelles and that these may resemble mitochondria (c.f. Gillman and Gillman, 1945). He prefers, at present, to reserve judgement on their actual identity and to call them siderosomes.

The histochemistry of the hæmosiderins is closely bound up with the demonstration of inorganic iron and methods for demonstrating

VIIIA. Human intestine (ileum). Activity of leucine aminopeptidase confined to the lining epithelium of the villi. L-leucyl-β-naphthylamide, Garnet GBC. × 150.



VIIIB. Normal guinea-pig granulation tissue before and after treatment with testis hyaluronidase. Toluidine blue. × 70.



VIIIC. Human stomach. Pyloric glands with orange-red granules in the argentaffin cells. Diazo method. × 600.

VIIID. Human vermiform appendix. Numerous macrophages in the lamina propria contain "pseudomelanin" pigment. Schmorl's ferric-ferricyanide—Carmalum. × 95.

[To face p. 656.

the latter are considered in Chapter XXIV, p. 683, and in Appendix 24.

Differential Diagnosis of Hæmosiderin. Theoretically hæmosiderin might have to be distinguished from any of the pigments already mentioned in this chapter and from hæmatoidin and the lipofuscins (see below). No difficulty is to be expected in making the diagnosis except where members of the lipid series of pigments contain iron. This is not a very frequent occurrence and when it occurs, usually in the lower range of oxidation, it seldom affects all the pigment in a particular location. If any doubt is felt about the nature of an iron-containing pigment associated with undoubted lipofuscin an attempt must be made to stain the granules by one or either of the lipofuscin reactions (PAS and Schmorl's reaction are especially useful), comparing the control slide with a serial slide stained by Perls' method.

Bile Pigments. Hæmatoidin and Bilirubin

Hæmatoidin was the name given by Virchow (1847) to the extracellular yellowish-brown crystals found in old hæmorrhagic foci. The relationship between this pigment and bilirubin remained uncertain until Fischer and Reindel (1923) showed that a sample of hæmatoidin from a cyst of the liver was identical with bilirubin. Rich and Bumstead (1925) obtained the same result with hæmatoidin crystals from other sources. Histochemically, therefore, the two substances are considered as " bile pigments " whereas morphologically it may still be reasonable to distinguish between them. The histological characteristics of the hæmatoidins, and their formation from blood and bile, were considered by Lignac (1923) in a comprehensive paper.

Since the work of Rich (1924) we know that hæmatoidin, like hæmosiderin, is formed intracellularly in phagocytes which have ingested red blood corpuscles. Unlike hæmosiderin, however, it does not remain there. Normally it is transferred into the blood stream and excreted by the liver but under certain circumstances, such as in old infarcts, its removal is prevented and it remains in the form of yellowish-brown crystals and amorphous granules, lying always extracellularly. Bilirubin appears in the gall bladder and bile ducts usually as bright orange-yellow amorphous masses and in the liver of obstructive jaundice in the form of bile thrombi.

The chemical and physical characteristics of the bile pigments make them relatively easy to distinguish histochemically. They are insoluble in water and in most fat solvents but moderately soluble in $CHCl_3$ or CS_2. These solubilities refer to unfixed or alcohol-fixed pigment ; formalin-fixed bilirubin and hæmatoidin are insoluble in $CHCl_3$ and CS_2. The bile pigments are not decolorized by oxidizing agents and although it is often reported that bilirubin is turned green

by 3 per cent. H_2O_2, I have seldom found this to be the case with tissue sections.

Oxidation methods for the demonstration of bilirubin and biliverdin have been studied particularly thoroughly by Kutlík (1957), who tested chromates, permanganate, iron alum, ferric sulphate, ferricyanide, peracetic and performic acids, alcoholic benzoquinone, H_2O_2, chlorate, periodic acid and nitrate, *inter alia*. Most of these reagents, in practice, were found to have undesirable qualities but, in addition to iodine, trivalent iron as ferric sulphate or iron alum gave reasonably controlled oxidation to green biliverdin. A further stage of oxidation to the reddish-violet bilipurpurine is best carried out with ferricyanide in alkaline solution at pH 7·2. These oxidation methods were regarded by the author as specific for bilirubin. They were negative with protein-bound bilirubin, kernicterus pigment, hæmatoidin and bilirubin-calcium compounds.

Bile pigments do not give primary fluorescence and they cannot be made to give the greenish secondary fluorescence with an unmodified Schlesinger's test as for urinary urobilinogen. Three further tests are described for the identification of bile pigments. These are the *methylene blue*, *Gmelin's* and *Stein's* tests.

The property of staining strongly with methylene blue has been shown by Reinhold and Fowler (1947) to be due to the formation of a compound of methylene blue with bilirubin, and this compound was successfully isolated. Unfortunately, from the histochemical point of view, nearly all the pigments we have to consider in this chapter share this property and stain strongly with methylene blue and other basic dyes. A positive test is therefore useless, but a pigment which does not stain with methylene blue is unlikely to be bilirubin or hæmatoidin.

Gmelin's reaction (Tiedemann and Gmelin, 1826) is usually carried out histochemically by the inclusion of concentrated HNO_3 under a coverslip and rapid examination under the microscope. A positive reaction is shown by a spreading greenish, and later blue or purple coloration. According to Lemberg and Legge (1949) the Gmelin reaction depends on the dehydrogenation of bilirubin to the greenish-blue bilatriene (verdin), and the green stage of the reaction is due to mixtures of bilatriene and unoxidized bilirubin. Similarly the blue stage is also due to a mixture, this time of bilatriene with violet pigments (purpurins) produced by its further oxidation to biladienediols. Finally the blue and violet compounds are oxidized to yellow choletelins. In the histochemical test, besides giving a display of colours, the original pigment is progressively dissolved. A successful Gmelin's reaction is completely diagnostic but a negative one does not exclude bile pigments, especially if the other tests are positive, since the reaction is apt to be capricious. With large amorphous masses of bilirubin it

invariably worked well but with small granules of hæmatoidin it is often negative. Crystalline hæmatoidin performs excellently.

Stein's (1935) test is supposed to depend on the oxidation of the pigment to green biliverdin by means of a solution of iodine. It works better with small granules of pigment than with the large masses and in my hands produces, at best, a darkening of the original golden yellow pigment to an olive-brown colour. The fact that this is not reversed by removal of the excess iodine with thiosulphate favours an oxidative mechanism for the change in colour but the possibility of some kind of combination with iodine cannot be ruled out. A negative Stein's reaction does not exclude the presence of bile pigment but a positive one, although iodine treatment may produce some darkening in pigments of the lipid series, is usually to be regarded as strong evidence in favour of its presence. Of the three colour tests described above, Gmelin's is the most specific for hæmatoidin and bilirubin. Gmelin's and Stein's tests together are sufficiently diagnostic in most cases, coupled with the absence of bleaching by H_2O_2 and other oxidizing agents.

Indirect and Direct Bilirubin. The difference between bilirubin and the bilirubin-like pigment excreted in the urine was first recorded by van den Bergh and Müller (1916) who observed that bilirubin and the serum pigment of patients with hæmolytic jaundice required ethanol before they would couple with diazotized sulphanilic acid. The pigment of bile and from the sera of patients with obstructive jaundice, on the other hand, would react directly. Cole and Lathe (1953) separated the direct and indirect types of pigment chromatographically and Cole *et al.* (1954) showed that the direct pigment had two components (Pigments I and II). Both of these are water-soluble and they appear in the urine in conditions such as obstructive jaundice and hepatitis where they form the main bile pigments of the serum. Bilirubin, on the other hand, is insoluble in water and in conditions such as acholuric jaundice, where it is the main bile pigment of the blood, the urine remains free of bile. Pigment II is the chief pigment of human bile and when coupled with benzenediazonium chloride it forms azo pigment B whereas bilirubin itself forms azo pigment A. Billing and Lathe (1956) and Billing *et al.* (1957) have now shown that pigment B is the ester glucuronide of pigment A. It is probable that these ester glucuronides are formed only in the liver.

In fixed tissue sections neither bilirubin nor hæmatoidin give a positive diazo reaction at acid or alkaline pH levels although Gedigk and Gries (1952) showed that bilirubin itself would couple directly in alkali. If cold microtome sections are treated (unfixed) with diazonium hydroxides a rose-red colour rapidly develops in certain areas especially in cirrhotic and other abnormal livers. It is possible that the water-

soluble bile pigments are lost during fixation. If they are preserved, due to protein binding, they are then non-reactive. The strong alkaline tetrazolium reaction (p. 114) of fixed bilirubin may be due to the glucuronide component and this reaction is recommended for the demonstration of bile thrombi in the livers of obstructive jaundice.

Hæmatins (Formalin, Malarial and Bilharzial Pigment)

Acid formalin pigment is a dark brown or black substance occurring in minute rhomboidal crystals and granules. It is produced in tissues rich in blood when these are fixed in formalin at an acid pH and is regarded as a hæmatin derivative. Lillie and Hershberger (1947a and b) and Hershberger and Lillie (1947) give excellent descriptions of its genesis and properties. It is easily distinguished histochemically by means of several characteristics, being doubly refractile, easily bleached by 3 per cent. H_2O_2 (30 minutes) and more easily bleached by 90 per cent. formic acid. It is extremely insoluble in strong acids but is extracted by alkalis, especially in alcoholic solutions. Saturated alcoholic picric acid removes it instantly. As emphasized by Lillie it is better practice to prevent its formation rather than to allow it to form and subsequently to remove it. In most cases formalin pigment does not form if the pH of the fixing bath is maintained above pH6·0. The presence of mercuric chloride, potassium dichromate, or picric acid, also inhibits its formation.

Malarial pigment is also regarded as a hæmatin and its histochemical properties are apparently identical with those of formalin pigment. Lillie (1948) discusses reported differences but considers their validity doubtful. Johnson *et al.* (1954) found that the histochemical reactions of the hæmatin pigment of schistosomiasis were identical with those of malarial pigment.

Aposiderin

This term is used to describe a brown granular iron-negative pigment which is not doubly refractile and which is extremely resistant to extraction. It is apparently formed from hæmosiderin by the action of acid fixatives which either remove the iron or render it inactive to Perls' and other tests. Its chief importance lies in the distinction between it and the lipofuscins of medium and high grades of oxidation. The best way out of the difficulty is to avoid the formation of aposiderin by the use of neutral or buffered neutral formalin. The former is usually adequate but it must be remembered that with some tissues the pH of the formalin solution, initially perhaps 7·4, may fall to 6·0 or lower. If distinction must be made, Schmorl's reaction is probably more reliable for the purpose, being negative with hæmosiderin and aposiderin.

The Lipid Pigments

All the pigmented substances discussed under this heading are derived mainly or partly from lipid precursors. They constitute a large class to which many names have been applied and, on the whole, the various subdivisions are characterized by rather indeterminate differences in reactivity with a number of histochemical tests. Most of the iron-free members of the class are considered to belong to the so-called wear and tear pigments (syn. brown atrophy, abnützungspigment, lipofuscin), others have been described as chromolipoid, hæmofuscin, cytolipochrome, lipochrome and ceroid. The iron-containing lipid pigments are sometimes regarded as variants of hæmosiderin and sometimes separately as cytosiderins.

The following members of the class of lipid pigments are described in this chapter: *lipofuscins*, *hæmofuscin* and *cytolipochrome*, *ceroid*, *pseudomelanosis pigment*, *iron-containing lipid pigment* and finally *lipochrome* and its contained carotenoids. No single histochemical property characterizes all these substances, and in many of them it is difficult or impossible to demonstrate lipid by means of the usual tests.

Lipofuscins

These pigments are derived from a number of lipid or lipoprotein sources and the process by which they are produced is at least partly an oxidation. Bensley (1947) described a red pigment occurring in the mitochondria and submicroscopic particles of guinea-pig liver cells. He compared this pigment with those produced by the autoxidation of phospholipids and unsaturated fats and found a close resemblance. Bensley suggested that the colour of the lipid pigments was due to the polymerization of aldehydes produced by oxidation. In this way the original easily soluble lipid, colourless unless it contains dissolved carotenoids, is oxidized to a very insoluble, often highly coloured product. The process varies with the nature of the original lipid and with the cell in which it takes place. It also varies with the extent to which protein forms part of the parent substance and on whether the constituent amino-acids of the protein are subsequently oxidized. Chemical studies carried out by Heidenreich and Siebert (1955) showed that the lipid content of an isolated lipofuscin (cardiac lipochrome) was 20 per cent. of the dry weight. The nitrogen content of the fat-free residue was 14·7 per cent. and hydrolysates revealed the presence of a number of amino-acids. The lipofuscins are the same as the chromolipoids of Ciaccio (see Chapter XI) which he regarded, similarly, as oxidation products of lipids. His chromolipoids, however, retained their sudanophilia in almost undiminished form, although

they became gradually more and more insoluble in fat solvents. They differed, moreover, in not reducing alkaline silver solutions. The term chromolipoid is short and accurate and it deserves almost equally with lipofuscin to be used for the description of the whole class of pigments which we are considering here. But because the more advanced stages of the pigments are no longer lipids in the accepted sense, it is probably better to call them fuscins, with the prefix lipo- to suggest their origin.

Views on the Histogenesis of Lipofuscins. My own views of the natural history of the lipofuscins can be expressed diagrammatically

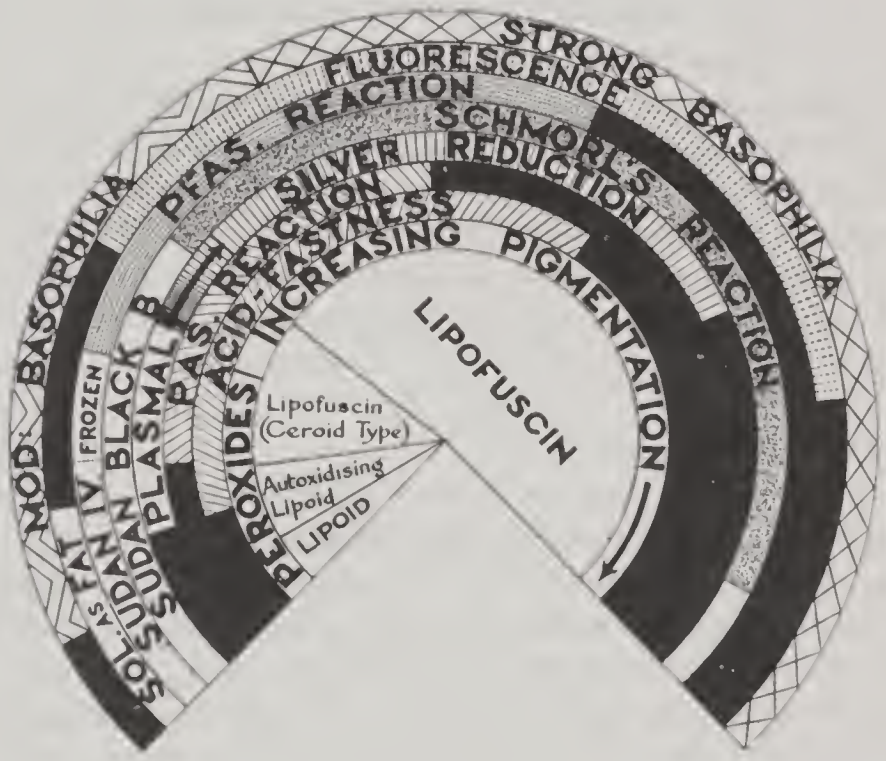


Fig. 207.

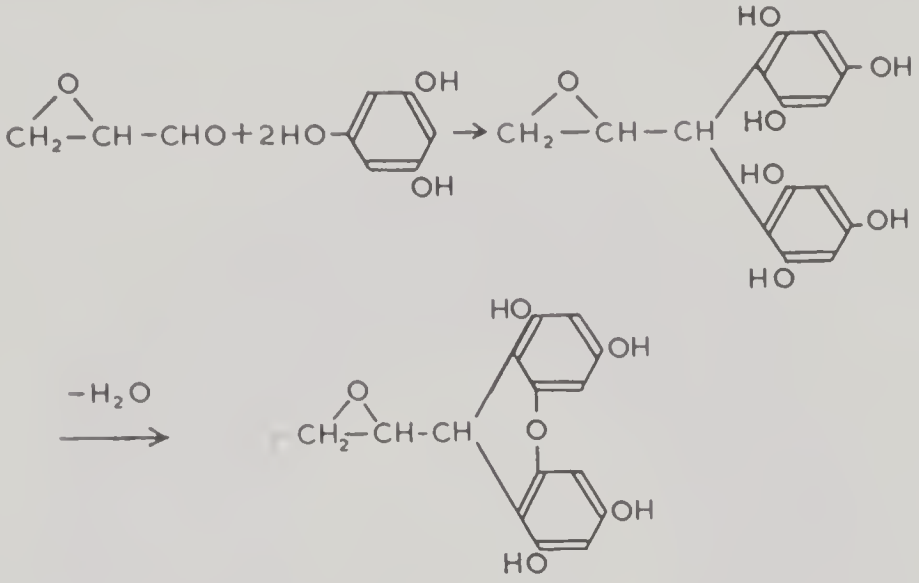
in terms of the histochemical tests which indicate their development (Fig. 207). At the lower left-hand edge of the diagram the lipid precursors of the lipid pigments are found in their original form. As oxidation begins their chemical and physical characteristics begin to alter and as it continues the characteristics of fat are replaced by those of partly oxidized fat. Pigmentation characteristically, but not inevitably, rises with the progress of oxidation ; some of the early reactions of oxidizing fat become negative and others become positive in their place. Thus, at the lower left-hand edge of the scale are the early products of the oxidation of triglycerides (column 2, Table 50) and of phosphatides (column 3). These are weakly basophilic, yet often actively fluorescent, possessing only weak reducing capacity, but strongly positive with Sudan black B and to a lesser extent with

Sudan IV and other fat-soluble dyes. The plasmal and PAS reactions are usually positive and fatty peroxides are present ; the pigment may or may not be acid-fast. It will be observed that ceroid, considered from the standard point of view in a separate section below, becomes an intermediate stage between autoxidizing lipid and lipofuscin. The typical, well-developed, brown lipofuscin pigment, which lies towards the right-hand end of the scale, is strongly basophilic, actively fluorescent and reduces both ferricyanide and silver solutions. It is regarded as highly oxidized lipid and the usual fat stains are always negative. The eosinophilic lipid globules in the pink epithelium of apocrine sweat glands, on the other hand, are to be regarded as hardly oxidized at all.

The point at which the lipid propigment becomes lipofuscin is not exactly determinable. It may logically be taken as the point at which a definite yellow colour becomes visible, remembering that in many cases a brownish-yellow colour is all that develops, although all the tests at the higher end of the scale become positive. Alternatively, it may be taken as the point at which the peroxide reaction of Dam (Glavind *et al.*, 1949) becomes negative. This reaction was shown by Dam and Granados (1945) to be due to oxidation products of highly unsaturated fatty acids and it disappeared during the production of lipofuscin pigment at a relatively early stage. The histochemical method (see Appendix 23, p. 927) was developed for the demonstration of peroxides appearing in lipid material during oxidation to acid fast pigment. The authors of the method had observed that, under suitable conditions, peroxidized fats and fatty acids reacted with a number of oxidizable substances (benzidine, leuco-malachite green and leuco-dichlorphenol-indophenol) to give coloured compounds. These reactions were accelerated by mild acidity and by heat and they were catalyzed by hæmin. In the method finally evolved fresh or formalin-fixed frozen sections were treated with a solution of leuco-3:5-dichloro-4:4′-dihydroxyphenylenediamine in the presence of hæmin for 3–5 minutes, when the substrate became oxidized to a red compound at sites containing peroxide groups. The specificity of this method has not yet been assessed but the localization of the dye within a variable proportion of fat cells, and sometimes in central zones only, seems to indicate that the explanation offered by the authors for the mechanism of the reaction is a true one.

In the second edition of his book on " Spot Tests " Feigl (1954) gives several reactions for the oxidation products of unsaturated fatty acids. As an alternative to the Dam reaction he suggests the use of 2,7-diaminofluorene, with hæmin as catalyst, for the detection of organic peroxides. This reaction I have found to be quite sensitive but the greenish-blue product is diffusible and localization is poor.

According to Feigl hydroxy fatty acids give a red colour with *sym* diphenylcarbazide in organic solvents and this reaction is sometimes weakly positive in sections containing oxidizing fats. A third reaction, this time for epihydrinaldehydes, is also occasionally positive in ceroid-type lipofuscins and in cardiac " lipochrome " if fresh sections are cut and stored for varying periods. This reaction depends on the development of a red colour when the aldehyde condenses with phloroglucinol in the presence of HCl.



Epihydrinaldehydes are volatile and cannot be expected to be present in large quantities in oxidizing fats. A negative reaction is therefore without significance, as indeed are negative reactions for organic peroxides and hydroxy fatty acids.

Enzymes in Lipofuscins. The presence of a non-specific esterase capable of hydrolysing Naphthol AS acetates was recorded by Gomori (1955) in various types of lipofuscin. I observed a similar non-specific esterase, especially in the lipofuscins of the nervous system, using indoxyl acetate methods (Pearse, 1955). A more extensive study was carried out by Gedigk and Bontke (1956) who found strong AS-type esterases and also acid phosphatases in a variety of lipid pigments. Gomori doubted the accuracy of localization in lipofuscins but I consider the presence of these enzymes as certain.

Significance of the Various Tests. At present we have little idea of the significance of the various tests which are positive in the case of the lipofuscins. Their solubility in fat solvents diminishes as their ability to dissolve fat stains diminishes, reflecting a progressive physical and chemical alteration. The number of acid groups available for combination with the basic dyes begins to rise almost as soon as oxidation commences and at this stage the plasmal reaction may become positive (see Chapter XII, p. 347). It remains positive only

for a short period although the comparable PFAS reaction persists for a somewhat longer period. The reason for the positive PAS reaction only in the intermediate stages of the oxidation of lipids is far from clear. It certainly does not mean, as many authors have concluded, that the lipid concerned belongs to the carbohydrate-containing glycolipid (cerebroside) group. We know (Chapter XI, p. 325) that aldehyde can be produced from non-carbohydrate-containing unsaturated phosphatides by the action of periodic acid, and we also know that such aldehydes cannot be produced from all phosphatide-containing tissue components by this method. Until much more work has been done upon the problem we can only say that, at a certain intermediate stage in the oxidation of lipids, Schiff-positive groups are revealed by brief oxidation with HIO_4.

At an early stage in the oxidative process reducing groups are formed which give a positive Schmorl's reaction and a weaker reduction of alkaline silver solutions. Beyond a certain stage of oxidation the latter effect becomes no longer noticeable but the reduction of ferricyanide increases in intensity and seldom drops to the point of extinction. Adams (1956) considered that the pigment produced in lipofuscins by the ferric-ferricyanide reaction was not Prussian blue but the green pigment ferroso-ferric ferricyanide (Lange, 1937) which is known as Prussian green. Lillie believes, however, that the green colour is a mixture of the yellow or brown of the pigment with Prussian blue. Details of Schmorl's reaction given in Appendix 23, p. 925, have been modified in the light of Adams' work.

The quality of acid-fastness depends to some extent on the way in which the test is performed and on the interpretation of what is acid-fast. Berg (1953) has shown that the acid-fastness of ceroid is dye specific. It is obtainable only when basic fuchsin is employed. I use a long Ziehl-Neelsen method (Appendix 23, p. 926) and regard the presence of any redness as positive. It is naturally less easy to distinguish this in the more highly coloured pigments so that, in the diagram, acid-fastness is indicated as falling off towards the right-hand end of the scale. A chemical explanation for this property has been suggested by the work of Reeves and Anderson (1937) on the chemistry of the lipids of the tubercle bacillus. Acid-fastness was thought to signify the presence of unsaturated fatty acids of high molecular weight. This test should not be used to distinguish between lipofuscin and ceroid pigment (see below).

A method for distinguishing melanins from lipofuscins was evolved by Hueck (1912) which depended on staining with Nile blue sulphate and subsequent treatment with H_2O_2 to bleach the melanins. This method was modified by Lillie (1956a) and followed by acetone extraction which leaves the melanins stained dark green and the lipo-

fuscins colourless. Lillie (1956b) investigated the mechanism of Nile blue staining of lipofuscins and found that two types of staining could occur. The first type he attributed to oil solubility and this occurred promptly with dilute solutions of Nile blue at pH levels down to 0·85 or lower. The process was rapidly reversed by acetone or alcohol extractions and could be repeated indefinitely. The second type of staining he attributed to formation of salt linkages. This took place with a number of basic dyes but was extinguished in every case at pH 3·0.

Hæmofuscin and Cytolipochrome

The name hæmofuscin was given by von Recklinghausen (1899) to a yellow, granular, iron-free pigment occurring in cases of hæmochromatosis together with very much larger amounts of hæmosiderin. Since by the majority of tests it reacted in the same way as lipofuscin, it was regarded as such by Hueck (1912). Mallory (1938), who described a basic fuchsin staining method for hæmofuscin, suggested that this pigment could be distinguished by its solubility in 5 per cent. H_2O_2. I do not find the iron-free pigment of hæmochromatosis, or the recognized lipofuscins, to be soluble in this reagent. There is not sufficient histochemical evidence to permit the retention of a separate fuscin pigment, derived from red cells, under the title hæmofuscin. Where red cell envelopes contribute to the formation of a lipid pigment, this has at all stages the characteristics of a lipofuscin. As such it should be regarded and described.

Gillman and Gillman (1945) described as cytolipochrome a pigment having the characteristics of hæmofuscin, which they found in the livers of pellagrins. This designation is entirely to be condemned. There is nothing in the nature of this pigment, as described, to warrant the invention of a new term, even if it had the merit of histochemical accuracy.

Ceroid

This pigment was first described, in the cirrhotic livers of animals maintained on inadequate diets, by Lillie *et al.* (1941, 1942) and by Edwards and White (1941). Edwards and Dalton (1942) later described a similar pigment occurring in the livers of mice treated with carbon tetrachloride. Wolf and Pappenheimer (1945) and Pappenheimer and Victor (1946) described the occurrence of a pigment, which they related to ceroid by virtue of its acid-fastness, in a wide variety of human tissues. They suggested that its development was due to deficiency of vitamin E. Endicott and Lillie (1944) established the main characteristics of ceroid pigment and the means of distinguishing it from other pigments such as hæmofuscin. It was described as occur-

ring in yellow globules 1–20 μ in diameter, usually in phagocytes, and as rims of basophilic, acid-fast material around large fat globules. It stained well with fat-soluble dyes in both frozen and paraffin sections. Lillie noted that the property of acid-fastness was shared by some of the lipofuscins, or as we should say, was shared by the lipofuscins at some stages of their development. This, of course, means that it cannot be used to distinguish between the two pigments. Lillie, however, maintained that distinction could be made by the negative reaction of ceroid with Schmorl's test. He did not (1948) mention the PAS reaction but Lee (1950) observed that ceroid in rat and mouse livers, produced by a variety of means, was variably positive. He observed in respect of this reaction that sometimes only a part of the pigment was stained and concluded that "although the pigment studied exhibited some similarity in acid-fast and fat-staining properties the variation in staining with iron reaction, periodic-acid-leucofuchsin, Schiff reaction, basic fuchsin and pyronin-methyl green indicated that ceroid may be a variable mixture of substances." McManus (1949) stated that ceroid gave a positive reaction for carbohydrate components by the Hotchkiss (PAS) and Feulgen reactions, "with as well as without acid hydrolysis." This last statement requires clarification since both reactions, without acid hydrolysis, are merely pseudoplasmal reactions. All lipids in an early stage of oxidation are likely to react in this manner. The PAS reaction, when positive, does not necessarily indicate the presence of carbohydrate either in ceroid or in lipofuscin, but the results obtained with blocking techniques suggest that some at least of the positive groups are due to the presence of polysaccharide. In the case of cardiac "lipochrome," however, which has some relationship to ceroid, the PAS reaction requires long oxidation to develop any intensity and this suggests (see Chapter IX, p. 238) that non-polysaccharide groups are responsible for the positive reaction.

My observations agree entirely with those of Lee in the matter of the variability of the pigment by various histochemical tests. I conclude, however, not only that ceroid is a mixture of substances but that it represents a typical lipofuscin in an early stage of oxidation. The acid-fast rims of large fat globules, which Lillie regarded as ceroid, occur in many other tissues besides liver. In particular, the lipid rims around the vacuoles of basophils in the human anterior pituitary are acid-fast, as are the PAS-positive vesicles described by me in the pituitary chromophobes (1948, 1949). Since these are not pigmented they are not to be regarded as lipofuscin but they can probably be regarded as ceroid. Alpert (1953), in a paper describing the hormonal induction of deposition of ceroid pigment in the mouse, summarizes the now firmly held belief that it is a fatty acid derivative

perhaps due to the failure of the normal cell mechanisms for breakdown of unsaturated fats. Alpert believes, and I agree, that the vacuoles which are so often seen in paraffin sections of ceroid masses represent part of the fat which has not undergone sufficient polymerization to be insoluble in fat solvents. In this material fatty peroxides can be demonstrated if cold microtome sections are used.

A high proportion of the lipoprotein envelopes of red cells, after fixation in mild oxidant fixatives such as Müller or Regaud, are strongly acid-fast. The substance responsible is not ceroid in the usual sense though it may well be chemically related to ceroid in possessing the same acid-fast groupings. Further confirmation of these views can be derived from the experimental production of a ceroid-like substance from erythrocytes, in rats, by Hartroft (1951), and from the work of Casselman (1951) on the histochemical properties of a similar artificially produced "ceroid." Acid-fast yellowish-brown autofluorescent pigments were observed to develop in Vitamin E-deficient rats and mink by Mason and Hartsough (1951) and in pigs by Robinson and Coey (1951). Tappel (1955) was able to produce yellowish-brown copolymers of ceroid type by the *in vitro* oxidation of unsaturated fats in the presence of proteins, especially hæmoglobin. The term ceroid originally applied only to the particular material in animal livers, produced by experimental means. Naturally-occurring pigments with similar characteristics, in man and other animals, can be referred to as lipofuscins (ceroid type), or as lipofuscins (early), or more simply as ceroid. This last term is now sanctified by common usage. Ceroid has been reported in atheroma (Burt, 1952), in the coronary arteries (Schornagel, 1956), in fluorocytes in the liver (Schmidt, 1953), and in hæmochromatosis (Weber, 1954). It has also been described in a further series of experimental studies by various authors (Williams and Aronsohn, 1953; Klein and Johnson, 1954; Wood and Yasutaki, 1956).

Pseudomelanosis Pigment

At one time there was a great deal of interest in the nature of this peculiar pigment, found almost exclusively in macrophages in the lamina propria of the large intestine and appendix. Hueck (1912), Henschen and Bergstrand (1913) and Dalldorf (1927) regarded the pigment as intermediate between melanin and lipofuscin, while Pick (1911), Lubarsch and Borchardt (1929) and Pick and Brahn (1930) considered it as a true melanin. Pick's final theory that aromatic protein degradation products, absorbed from the large intestine, were converted into melanin within the connective tissues by a tyrosinase-like enzyme, was accepted by Stewart and Hickman (1931). Speare (1951) has revived the "anthracene" purgative theory of pseudo-

melanosis by reporting a series of cases which, without exception, were associated with chronic medication with *cascara sagrada*. This substance contains anthraquinone glucuronides. Some German authors, apparently on the basis of a reported positive reaction by both Prussian and Turnbull blue tests (Appendix 24, p. 931), have considered pseudomelanin to be a hæmosiderin, altered by the action of H_2S. All the pigment, in those examples of pseudomelanosis of the colon and appendix which I studied while investigating this particular problem, was negative by both the Turnbull and Prussian blue reactions and I agree with Stewart and Hickman's decision that the pigment is not derived from hæmoglobin. Piringer-Kuchinka (1952) considered pseudomelanin as an "exogenous pigment" and a similar view is taken by Hieronymi (1954). Pseudomelanosis pigment is shown in Figs. 205 and 208, and also in Plate VIIID, p. 656, where its reaction with Schmorl's ferric-ferricyanide is recorded.

Iron-containing Pigments

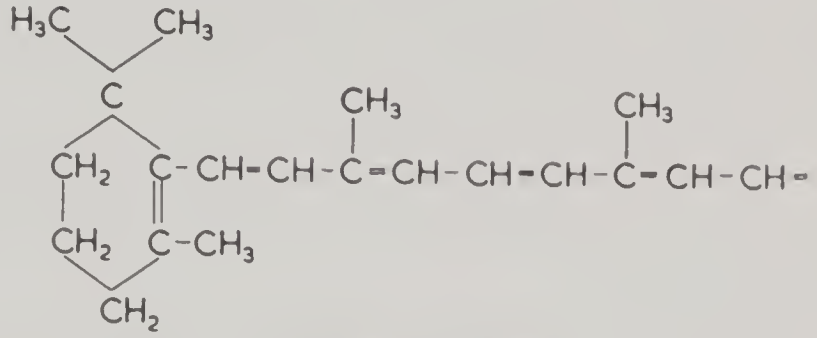
This is not the point at which to consider the differential diagnosis of the hæmosiderins ; it is necessary, however, to mention the occasional occurrence in collections of lipofuscin pigment, of almost identical but non-fluorescent, iron-containing, granules. It is possible to regard these as true hæmosiderin but, more probably, they are due to the combination of iron salts with the lipofuscin at some stage in its formation.

Lipochromes

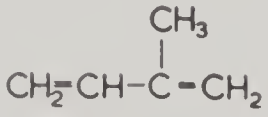
By definition these are lipids containing in solution coloured hydrocarbons of the widely-occurring carotenoid series. The presence of dissolved carotenoids is usually obvious enough, in fresh tissues, because of the brilliant yellows and reds which they impart. Some examples are the corpora lutea of women and the corpora lutea and corpora rubra of cows, the ordinary body fats of many animals and the adrenal cortical lipids. The carotenoids are soluble in light petroleum, benzene, xylene, chloroform, ether, alcohol, acetone, etc., and they are largely dissolved out during the preparation of paraffin sections. For this reason we are not usually concerned with lipochromes except in fresh or formalin-fixed frozen sections and in freeze dried material. Carotenoids combined with protein may remain sufficiently firmly attached to withstand the process of paraffin embedding, but little is known of the occurrence of this particular type of chromoprotein in mammalian tissues.

The Carotenoid Pigments. Whatever the nature of the lipid part of the lipochrome, and this may vary within wide limits, the diagnosis of the whole depends on the demonstration of carotenoids. The

methods available for this purpose are discussed below. According to Karrer and Jucker (1950), approximately eighty natural carotenoids are now known. They are all closely related chemically and belong to the class of polyenes (polyisoprenes) whose chief characteristic is the possession of a large number of conjugated double bonds. Below is illustrated the formula of half the molecule of β-carotene ; the other half being the mirror image of that given. The whole molecule consists of eight isoprene residues linked together, not all in the same way. The formula of isoprene is also given.



β-carotene (½ molecule)



Isoprene

As far as animals are concerned, the carotenoid pigments are derived entirely from the diet and, originally, from vegetable sources. They are partially absorbed from the intestine and partially excreted unchanged. Three carotenoids in particular have been recorded as occurring in human serum (Willstaedt and Lindquist, 1936) ; these are carotene, lycopene and xanthophyll. The same three have been identified in human liver (Zechmeister and Tuzson, 1936). They are known to act as sources of vitamin A, as first suggested by Steenbock *et al.* (1921) ; later Karrer *et al.* (1931, 1933) showed that β-carotene could be converted by the uptake of water into two molecules of vitamin A. Carotenoids also play a part in the process of vision. Rhodopsin (visual purple) is considered by Wald (1935) to be a carotenoid-protein complex, and retinene, which also occurs in the eye, is the aldehyde of vitamin A.

Since the carotenoids are extremely soluble in fat solvents they are seldom found in paraffin sections except where they occur as chromoproteins. When dissolved in lipids, however, they are insoluble in water, glycerol, formalin, and dilute acids and alkalis ; they are relatively insoluble in ethanol and methanol. In formalin-fixed frozen, or in freeze-dried sections they remain *in situ*. Since β-carotene and

the other carotenoids are autoxidized in the presence of atmospheric oxygen to colourless compounds, material for histochemical tests should be examined as soon as possible. Judging by the progressive reduction in colour of the adrenal cortical lipids, autoxidation also occurs in formalin.

The Histochemistry of the Carotenoids. Carotenoids have been demonstrated histochemically by means of certain colour reactions. They give green, blue or violet colours with a variety of strong acids such as sulphuric, hydrochloric, phosphoric, perchloric, formic, and trichloroacetic, and blue colours with the chlorides of antimony and arsenic. In practice it is usual to carry out the reaction with sulphuric acid or the Carr-Price test with antimony chloride in chloroform. Neither reaction is specific though they both might well be made more so by examining the absorption spectrum of the resulting blue compound (maximum 590 Mμ with β-carotene). With both tests a weak and transient colour is produced. Although the colours produced by the action of antimony chloride on the carotenoids are usually unstable those produced with the iodine derivatives are said to be more stable. Lison (1936) mentions the demonstration of carotenoids by forming such iodine derivatives and examining sections in reflected light for a metallic blue sheen.

The carotenoids and vitamin A exhibit green fluorescence in ultraviolet light and this property has been utilized in the extensive researches of Popper (1941, 1944) on the distribution of vitamin A in human tissues. According to this author it is possible to distinguish the green fluorescence of carotene, which fades very slowly, from the identically-coloured, rapidly-fading fluorescence of vitamin A. Ball and Morton (1949) have described the reactions of the latter with sulphuric and phosphoric acids. With conc. sulphuric acid at 0° a blue compound is produced (absorption maximum 620 Mμ) which quickly fades to a series of red compounds (absorption maxima 465, 520 and 580Mμ). At the lower temperature fading of the blue component is much less rapid than at room temperature. Ball *et al.* (1949) give similar data about retinene and, in addition, they describe the combination of this substance with amines. The reaction is usually slow but with the aromatic amines coloured compounds are quickly produced. Aniline (in excess in acid solution) gives a red compound (absorption maxima 490–435 Mμ).

In routine histochemistry the carotenoid pigments are of very little importance though in research their interest is obviously greater. The results obtained with the usual reactions (Carr-Price and conc. sulphuric acid) are disappointing and the knowledge gained in biochemical investigations of the type quoted above has not yet found histochemical application.

The Differential Diagnosis of Tissue Pigments

Table 50 shows the reactions of various types of lipid pigment and propigment and, for comparison, the reactions of melanin, hæmosiderin, hæmatoidin, argentaffin cell granules, adrenal chromaffin granules and of " pseudomelanin " in the lamina propria of the colon and appendix. The pigments in the first nine columns are all of lipid origin and in the remaining columns appear the other pigments dealt with in this chapter. The table may therefore be used as a reference sheet of reactions in the diagnostic histochemistry of mammalian pigments.

At first sight, the results appear to show only a general similarity between all the lipo-pigments and a number of minor points of variance. A most important observation, which cannot be shown clearly in a bald record of responses to the various tests, is that in any one situation the individual granules of lipo-pigment often differ both in appearance and reaction. This point is best illustrated by example. In the posterior pituitary gland of man there accumulate numerous intracellular pigment granules varying in size from 0·2 to 1 μ, and in colour from yellow to brown. Similar pigment granules occur in many other sites listed in the table, notably liver, adrenal and ganglion cells, and they also occur in numerous sites not listed. In the case of the neurohypophysis about half of the granules, mainly the larger and less pigmented ones, can be stained with Sudan black B in paraffin sections; they are PAS-positive, reduce ferricyanide (Schmorl) and alkaline silver solutions, and are only weakly basophilic. The remainder, consisting of the smaller and more pigmented half of the granules, stain strongly with basic dyes and are acid-fast ; they are less strongly reducing towards ferricyanide and silver solutions, and they are Sudan black and PAS-negative. In my opinion these differences reflect not the existence of two different types of lipid pigment, but of two main varieties of the same pigment, lipofuscin.

The pigments listed in the first three columns of the table are shown by their content of peroxides to be in an early stage of autoxidation, and the positive plasmal reaction indicates that acetals are among the reaction products of this process. All three can be shown with fat-soluble dyes to contain at least a proportion of lipid. The pigments in columns 4–8 inclusive usually still contain lipid demonstrable by this means and the presence of unsaturated lipids in nearly all of them is revealed by the positive PAS and PFAS reactions. The behaviour of heart muscle " lipochrome " (column 4) is anomalous and this material cannot be fitted into the same precise group (of intermediate lipofuscin) as the others.

The reactions of " ceroid " pigment show that it can conveniently

TEST OR PROPERTY	LIPO-PIGMENTS									OTHER PIGMENTS (FOR COMPARISON)					
	Apocrine gland globules	Perirenal fat (early oxidation)	Lipid in macrophages	Heart muscle "lipochrome"	Posterior pituitary	Ganglion cells	Liver cells	Adrenal (reticularis)	Ceroid liver	Melanin (melanoma)	Hæmosiderin	Hæmatoidin	Argentaffin cell granules	Pseudomelanosis (appendix)	Chromaffin (adrenal medulla)
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
Colour	Pale yellow	Pale yellow	Yell. to brown	Gold-brown	L. to D. brown	Yell. to D. brown	Light brown	Gold-brown	Yellow-brown	Brown to blaek	Yell. to brown	Yellow-brown	Pale yellow	Yell. to brown	Brown
Eosinophilia	+	±	−	−	−	−	−	−	−	−	−	−	+	−	−
Basophilia	−	+	+	weak +	+ v.	weak + to +	+	+	+	+	weak + to −	−	−	weak + to +	weak +
Aeid-fast (long Z-N)	−	−	weak +	+ v.	+ v.	+ v.	+	+	+	oee. +	−	−	−	−	−
Sudan blaek	+	+	+ to −	±	±	±	±	±	+	−	−	−	−	−	−
Sudan IV (frozen)	mod. +	mod. +	mainly −	mod. +	usually −	−	−	−	weak +	−	−	−	−	−	−
PFAS	+	+	+	+	+ v.	+ v.	+ v.	+ −	+ v.	−	−	−	−	±	−
PAS	−	−	±	−	±	±	±	±	±	oee. +	−	−	−	±	−
Plasmal	part +	part +	part +	−	−	−	−	−	−	−	−	−	−	−	−
Alkaline silver	weak +	weak +	weak +	−	weak +	weak +	weak ±	−	weak +	+	−	−	+	±	+
Sehmorl	weak +	weak +	weak +	weak + to −	+ to weak +	str. +	+	+	±	+	−	−	+	±	str. +
Gmelin	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
H_2O_2 (bleaehing)	−	−	−	48 +	48 +	48 +	48 +	48 +	−	24 to 48	−	−	−	6–8 +	1–2 +
Diazo (alkaline)	−	−	−	−	−	−	−	−	−	−	−	−	+	Deep yell. brn.	Deep yell. brn.
Diazo (aeid)	−	−	−	−	−	−	−	−	−	−	−	−	+	Yell. brn.	Yell. brn
Gibbs'	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Fluoreseenee (U.V.)	Gold-yellow	Brown-gold	Yellow	Red-brown	Green-yellow	Gold yellow	Dull brown	Weak to nil	Gold-brown	−	−	−	White-yellow	Gold-yellow	−
Chrome hæmatoxylin	−	−	−	+	+	+	+	+	−	−	−	−	−	+	−
Peroxide (Dam)	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−

The following abbreviatons are used : yell., yellow ; wh., white ; brn., brown ; blk., blaek ; L., light ; D., dark ; v., varies ; mod., moderate ; str., strong. In the case of bleaching by 3 per eent. hydrogen peroxide the numbers refer to the approximate time (hours) of treatment neeessary to deeolorize the darkest form of the particular pigment listed. The referenee 48 + means more than 48 hours. Where the symbols ± or + − oeeur in the Table, this does not mean the pigment as a whole may be either positive or negative but that at the same site it is partly positive and partly negative ; the upper symbol indieates predominance. Similarly, the symbol + v. means that the pigment is usually entirely positive but that some granules in the same situation in the seetion may be negative.

be considered as an early lipofuscin in the stage of autoxidation, and that none of the tests performed can be used to distinguish it from other members of this group. This does not mean that the pigments in columns 4–9 have identical origins and identity ; only that we are at present incapable of demonstrating any differences which may exist between them.

Of the pigments in the comparison group (columns 10–15) only one resembles the lipo-pigments in its reactions. This is the so-called " pseudomelanin " found in the intestinal wall. Where two symbols or descriptions appear in column 14 the upper one refers in each case to the pigment situated at the mucosal border of the lamina propria, and the lower symbol to the pigment in cells deeply situated just under the muscularis mucosæ. I assume that the former is the younger and the latter the more mature pigment. The newer material occurs in the form of yellow granules which are brilliantly fluorescent, strongly reducing towards both ferricyanide (Fig. 205) and alkaline silver solutions, weakly basophilic, strongly PAS-positive (Fig. 208) and positive also by the PFAS reaction. The older pigment is darker in colour, sometimes approaching black, less strongly fluorescent, and much less strongly reducing. The oldest pigment is PAS and PFAS-negative.

These characteristics are those of a lipofuscin containing lipid, possibly small in amount and combined as lipoprotein, oxidized sufficiently at all stages to give negative results with the Sudan dyes. The results do not suggest that the pigment is a true melanin since, in those critical positive reactions which it shares with lipofuscin, melanin never shows the same range of variability. The rapid bleaching of the intestinal pigment with H_2O_2 also suggests that it is not a true melanin, but only one reaction gives any indication that it may not be a true lipofuscin. This is the diazo reaction which invariably increases the colour of the granules to a deep yellow, and even to a reddish-brown in some instances. This is in contrast to the behaviour of true lipofuscin granules whose colour, at all stages, is unaffected by diazonium salts or their breakdown products. Chromaffin granules, on the other hand, give a reaction almost identical with that given by pseudomelanin. Even if the increase of colour is due not to coupling with the diazonium salt but to a strong affinity for its coloured breakdown products in alkaline solution, the similarity between pseudomelanin and chromaffin suggests that Pick's hypothesis of aromatic breakdown products offers the best explanation in this case. Staining with a diazotate (inactivated by treatment at 60° and pH 9·2 for 3 hours) scarcely affects either pseudomelanin or chromaffin granules but this control experiment does not exclude the possibility that intermediate breakdown products of the diazonium salt may be

responsible. I, therefore, regard pseudomelanin as belonging to the class of lipofuscins but containing an additional element derived from lipid-soluble phenolic oxidation products.

The reactions recorded for the remaining pigments (columns 10, 11, 12, 13 and 15) show that no difficulty is experienced in recognizing the two belonging to the hæm group (hæmosiderin and hæmatoidin) and in distinguishing them from the others in the table. The tyrosine-tryptophan group of pigments (columns 10, 13 and 15), on the contrary, is shown to possess a number of reactions in common with the lipo-pigments. Those reactions usually thought to be diagnostic for one or another of the pigments (e.g. Schmorl's reaction for lipofuscin and the alkaline silver reaction for melanin) are obviously unspecific. In each case, however, there is at least one negative or positive reaction to distinguish the members of the tyrosine group from each other and from the lipo-pigments in the middle and upper ranges of oxidation. Melanin, except in melanophages, never gives a positive PAS or PFAS reaction and this feature, and the shorter time required for bleaching, distinguish it from the lipofuscins. It can also be distinguished by the alkaline tetrazolium reaction which is positive only with lipofuscins and by methylene blue extinction (MBE). Melanins are basophilic down to and sometimes below pH 1·2. Lipofuscins seldom bind methylene blue below pH 1·8. Chromaffin granules are distinguished from the latter, and from melanin, by an extremely short bleaching time, and by the yellow-brown colour which they give with the diazo reaction. Argentaffin granules give a true positive diazo reaction (red) and a positive Gibbs' reaction, and they differ on this account from the other members of the group, and from lipofuscins.

In spite of the above criteria, however, the histochemical diagnosis of pigmented substances in tissue sections is not always easy or straightforward. It will not be so until some of the many gaps in our knowledge of the mechanism of pigment formation are closed.

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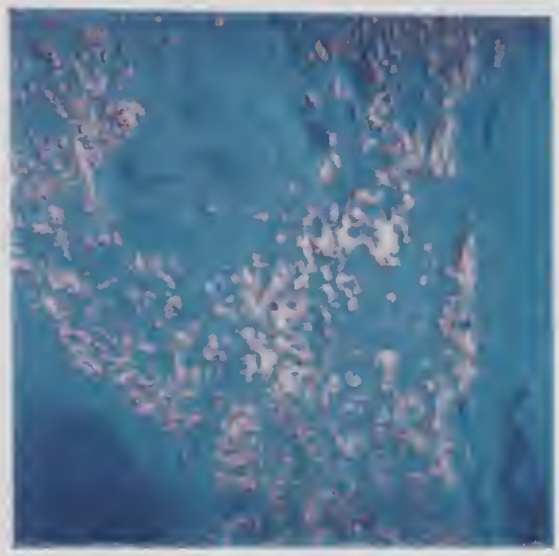
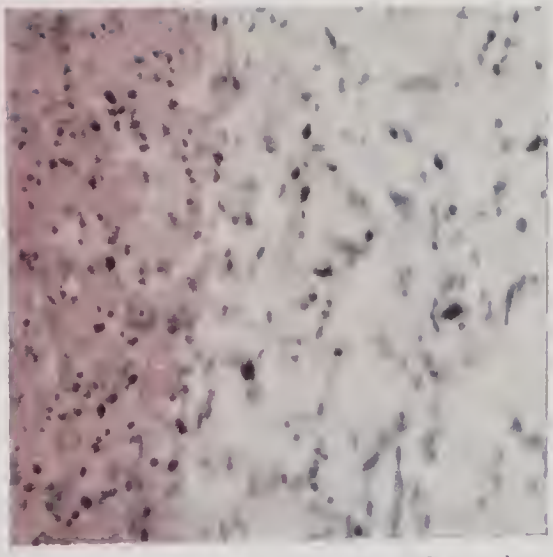


IXA. Rat lung containing partly solubilized deposits of aluminium dust. These show strawberry-pink fluorescence. Solochrome dark blue BS. × 385.

IXB. Rat lung containing similar deposits. Solochrome azurine. × 250.



IXC. Rat lung as in Plates IXA and B. Acid Solochrome cyanine. × 300.

IXD. Human lymph node. Crystalline doubly refractile beryllium deposit surrounded by zone where a soluble beryllium salt is combined with protein. Alkaline Solochrome azurine. × 200.

[*To face p.* 681.

CHAPTER XXIV

INORGANIC CONSTITUENTS

Introduction

From the point of view of the routine histochemist, iron and calcium are the two most important inorganic constituents of tissue sections, with phosphate and carbonate closely following. This is because satisfactory and simple methods exist for their demonstration. From the physiological point of view, sodium, potassium and chloride are far more important than the constituents mentioned above but for these the ordinary methods of histochemistry have in the past been largely unsatisfactory.

Numerous chemical techniques exist for the localization in the tissues of other metallic ions, e.g. gold, silver, platinum, mercury, lead, copper, nickel, aluminium, zinc, palladium, uranium, bismuth and arsenic. With most, if not all, of these metals it is necessary first to introduce them into the tissues, and their subsequent " specific " demonstration depends largely on the fact that one knows what to expect. There are two alternative ways in which the problem of localizing inorganic ions in the tissues may be undertaken. These are (1) micro-incineration and (2) autoradiography. The former is capable of giving accurate information about the total distribution of certain minerals in tissue sections but, unless emission histospectrography is used in conjunction, only calcium, magnesium, iron and silicon can be distinguished. The amount of information which can be obtained from the simple micro-incineration of a tissue section is limited, although by the addition of histospectrographic techniques such as those employed by Policard (1933) and by Scott and Williams (1935), and of analytical electron microscopy (Scott and Packer, 1939), quantitative information about very small quantities of minerals becomes available. These additional techniques, however, take the practice of micro-incineration out of the hands of ordinary histochemists and the method and its technical details are not further considered in this book. An excellent review by Horning (1951) appears in Bourne's *Cytology and Cell Physiology*, in which the status of micro-incineration in modern histochemistry is fully discussed. The method itself is dealt with by Horning, and also by Glick (1949).

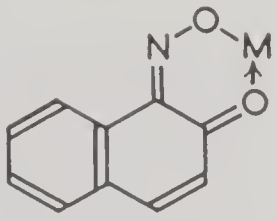
The applications of autoradiography are discussed in Chapter XXVII. This method cannot give information about minerals of the type given by micro-incineration but only about the handling in the tissues of

various elements introduced in radioactive form. This kind of information, being functional and not static, is likely to be of greater importance than the other. In this chapter, however, only the ordinary histochemical methods for inorganic elements will be discussed.

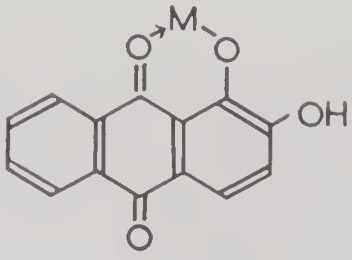
Histochemistry of Inorganic Cations

Lakes and Chelates

Many histochemical methods for inorganic elements, especially metals, depend on the formation of coloured compounds with coloured or colourless reagents. In the pigment and dye industry the word lake (Sanskrit, *laksha*, a hundred thousand) has been used very loosely, but in histological practice it usually means an adsorption compound formed by union of a dye with a metal or metal salt. Lake-forming metal salts, when used as histological reagents, are referred to as mordants (Latin, *mordere*, to bite) from an old idea that they attack the material being dyed before causing the dye to adhere. In this chapter the word lake has been used to mean an adsorption compound of metal salt with dye, particularly when the nature of their union is a simple electrovalent salt linkage, or when it is unknown. The term chelate, on the other hand, is used to refer to non-ionic metallic linkages of the types illustrated below :



1—Nitroso—2—naphthol



Anthraquinone

where the metal is held by strong covalent (co-ordinate) linkages (35–100 k.cal. per mole). A chelator, therefore, is a compound capable of forming a metal chelate, like those shown above, where M may be any of a number of metals and where the atoms of the metal may be bound in a ratio of 1 : 1 of the chelator or in some other ratio. For a molecule to function as a chelator it must (1) possess at least 2 atoms which can attach to the metal, and (2) the atoms must be joined so that their valence bonds are at their natural angle. (The natural angle of the C atom = 108, i.e. the angles of a pentagon.) The most usual atoms which form the electron donors of a chelator are O, N, and S.

The literature on colour lakes has been thoroughly reviewed by Blumenthal (1946) and also by Venkataraman (1952) in his two volumes on the "Chemistry of Synthetic Dyes." The whole subject

of the coordination compounds is covered by Bailar (1956) whose book should be consulted by those interested in the theoretical aspects of chelation.

Methods for Inorganic Iron

There are three classical methods for this purpose : the Prussian blue (Perls, 1867), Turnbull blue (Schmeltzer, 1933), and iron sulphide (Quinke, 1868) methods. Three other tests which are available are the dinitrosoresorcinol methods of Humphrey (1935), the 8-hydroxyquinoline method of Thomas and Lavalloy (1935) and the Mallory (1938) hæmatoxylin method. The Prussian blue reaction depends on the production of ferric ferrocyanide when ferric ions (Fe^{+++}) in the tissues react with ferrocyanide in acid solution. The Turnbull blue method depends, in its simplest form, on the production of ferrous ferricyanide by the reaction of tissue ferrous ions (Fe^{++}) with ferricyanide. Since, however, iron in the tissues is almost invariably present in ferric form, the Turnbull blue reaction is usually performed after the manner of Schmeltzer (Tirmann-Schmeltzer), that is to say, following conversion of Fe^{+++} to Fe^{++} with $(NH_4)_2S$.

The three best reviews on the demonstration of inorganic iron in the tissues are those of Gomori (1936), Lillie (1948) and Bunting (1949). The last two authors agree that neutral 10 per cent. formalin is the best fixative for the purpose and that by its use more iron is preserved, with less diffusion, than by other and especially by alcoholic fixatives. Bunting confirmed the earlier observations of Gomori that the particles of iron demonstrated by the Turnbull blue method were larger than those produced by Quinke's method. Both were larger and more distorted than the Prussian blue particles. This last reaction, moreover, has the highest sensitivity of the three methods (0·002 γ of Fe^{+++}, according to Lison, 1953 ; 0·1 γ of Fe^{+++} according to Feigl, 1954) and it certainly reveals more iron than the other two.

Treatment with dilute acid is an essential preliminary in the performance of the Prussian blue reaction since by this means the ferric ion is liberated from unreactive loose combinations with protein, such as hæmosiderin. Iron combined in the tissues as hæmoglobin (masked iron) is more strongly bound and cannot be released by acid treatment. The application of 100 vol. H_2O_2 (30 per cent.) for a short period makes some of this masked iron available for Perls' reaction. In performing the reaction the two reagents are invariably employed simultaneously, since, if treatment with ferrocyanide follows treatment with acid, diffusion of iron is considerable. Various investigators have used different concentrations of acid and ferrocyanide. Gomori (1936) used equal parts of 20 per cent. HCl and 10 per cent. $Na_4Fe(CN)_6$; Lison (1936) used equal parts of 2 per cent. (v/v) HCl and 2 per cent. (w/v)

ferrocyanide, and Lillie (1948) equal parts of 5 per cent. acetic acid and 2 per cent. $K_4Fe(CN)_6$, at a temperature of 80°. Bunting (1949) varied the concentration of both acid and ferrocyanide and obtained the best results with those employed by Lison. Because of its high degree of sensitivity and specificity the Prussian blue method of Perls, using the concentrations advised by Lison, is the method of choice for the histochemical demonstration of hæmosiderin iron and other protein-bound forms.

The Quinke method gives a green or brownish-black colour to the large particulate deposits of hæmosiderin but the finer particulate and the non-particulate deposits are not distinguishable by this method. Much more sensitive is the $(NH_4)_2S$-dinitrosoresorcinol method of Humphrey, which depends on the formation of a greenish-black ferrous iron-lake. This method works well and may be employed if, for any reason, complete freedom from iron-containing reagents is necessary, as it might be in a critical histochemical investigation. The Humphrey method used to have the advantage of permanency over the Prussian blue reaction, for mounting in Canada balsam, but since the introduction of synthetic mounting media this advantage has largely disappeared.

The Tirmann-Schmeltzer reaction is not recommended. Lillie has shown that it demonstrates only a small portion of the available iron present. If sulphide-treated sections are placed in acid ferrocyanide instead of in acid ferricyanide, a perfectly good Prussian blue reaction occurs.

The two remaining methods depend, like that of Humphrey, on the formation of a coloured iron-lake. With the Thomas and Lavalloy method I have been completely unable to obtain a satisfactory result and, indeed, the rationale of the method requires explanation. If ferric iron is released from combination with protein by the 4 per cent. acetic acid of their hydroxyquinoline solution, the ferric hydroxyquinoline complex so formed may not be present in sufficient concentration for its solubility product to be exceeded locally. Thus a precipitate may not occur. Thomas and Lavalloy sought to establish conditions suitable for precipitation, after the initial reaction was ended, by the addition of 25 per cent. ammonium hydroxide. In my experience this produces a diffuse precipitate of hydroxyquinoline together with a few particles of the ferric salt. The latter are not related to iron-containing structures and one would scarcely expect them to be. Glick (1949) suggested that the ferric hydroxyquinoline produced by this method might be identified by its characteristic fluorescence, but the precipitate of this substance formed in the Friedenwald and Becker method for β-glucuronidase (see Chapter XVII, p. 498) does not give a satisfactory fluorescence in the tissues.

Mallory's method for iron is sensitive but of low specificity, since a blue-black hæmatoxylin lake is formed with many other metals, including calcium. It is possible to exclude most of these without much difficulty and the method may still be useful, on occasion, for inorganic iron. Hæmosiderin usually stains only brown, but if it is first treated with $(NH_4)_2S$ the blue-black lake is produced. The interstitial and connective tissue varieties of diffuse "hæmosiderin" give the blue-black colour without sulphide treatment.

The application of Perls' reaction to deposits containing inorganic iron is illustrated in Fig. 209, p. 688. This and the adjoining figures show the application, to formalin-fixed paraffin sections of a granulomatous lymph node, of some of the reactions described in this chapter. In each case giant cells are present containing doubly-refractile crystalline material (Fig. 210), and Perls' reaction (Fig. 209) shows that the crystals also contain iron. The presence of phosphate in the crystals is not illustrated but in Fig. 211 it is shown in the giant cells by a modified Serra technique (see below). Fig. 212 shows the application to the giant cells of the Alcian blue method of Steedman (see Chapter IX, p. 259). The crystals are strongly stained with the dye and the cytoplasm of the giant cells is pale blue. Mucin was not demonstrable in the cells by any other method and the most likely explanation of this finding is that Alcian blue, like other phthalocyanins, forms a lake with calcium and other metals (see section on Calcium, below). Organically-bound iron does not stain with Alcian blue and iron-containing crystals of the type described above, which occur in a variety of granulomatous conditions, including sarcoidosis, do not stain invariably by this method, although they are always positive by Perl's reaction.

Ferritin

This soluble iron-containing protein was first isolated from horse spleen in 1937 by Laufberger, who showed that it contained 17–23 per cent. of iron. It consists (Granick, 1951) of a homogeneous protein called apoferritin (M.W. 460,000) and an iron-containing part said to be present in the form $(FeOOH)_8.(FeOPO_3H_2)$. This iron can be removed from the protein by the action of reducing agents such as hydrosulphite. Both apoferritin and ferritin are precipitated in crystalline form by cadmium sulphate (Granick, 1946), and this forms the basis of one histochemical method for their demonstration (see below). Using rabbits, Granick (1943) prepared anti-horse ferritin antibodies which precipitated both ferritin and apoferritin and, by means of the precipitin reaction, he showed that ferritin was present in spleen, bone marrow, liver, testis, kidney, pancreas, ovary and lymph nodes. These organs are listed in order of diminishing ferritin content. In the case of the

dog Mazur and Shorr (1950) calculated the γ of ferritin N per gram of wet tissue as follows : liver, 97 ; spleen, 94 ; bone marrow, 16 ; kidney cortex, 17 ; pancreas, 6 ; skeletal muscle, 4.

Ferritin is now regarded as the main storage form of iron, having thus taken the place of hæmosiderin, which is to be considered as the storage of iron in excess. It is formed in the mucosa of the gastro-intestinal tract when iron is given by mouth and in other tissues when, for instance, iron is set free by hæmorrhage or hæmolysis. In general, tissues which have a high hæmosiderin content contain large amounts of ferritin. In the placenta iron is transported from mother to fœtus against a concentration gradient, and Vosburgh and Flexner (1950) have suggested that a special transport mechanism exists. Nylander (1953) succeeded in demonstrating a soluble form of ferric iron in the villous portion of the visceral yolk sac membrane in the rat by means of the Prussian blue reaction on fresh tissues. In the visceral yolk sac, and in the allantoic placenta, he was able to demonstrate the typical isotropic, octahedral yellowish-brown crystals after incubation in cadmium sulphate. This technique was also used by Diezel (1955) to demonstrate the presence of ferritin in the globus pallidus of the human brain.

Although it is strictly a method for demonstrating the protein part of ferritin, it is probable that the cadmium sulphate technique, together with the Prussian blue reaction, will continue to be used for the localization of ferritin. For this reason it has been considered in this chapter and details are given in Appendix 24, p. 932.

Methods for Calcium

These methods are divisible into two classes : those which are more or less specific for calcium, and those which really indicate the phosphate or carbonate with which the calcium is often combined. Calcium occurs in the animal body in a number of different forms. It may be present as a soluble salt (chloride, sulphate or lactate) in the blood serum, in an ionized or ionizable form in combination with serum or other proteins, in masked form, or in an insoluble form as deposits of phosphate or carbonate, or of the two together.

Soluble Forms. These have been demonstrated by treating fresh tissues with soluble salts of oxalic or tartaric acids, the insoluble calcium salt being deposited *in situ* in crystalline form. According to Lison (1935, p. 156), the sensitivity of these methods is high ; he gives a figure of 0·06 γ for the oxalate method. Crétin's method, described below, can also be used for the demonstration of soluble calcium salts in fresh tissues. Rabl (1923, 1926) fixed thin slices of tissue in a mixture of equal parts of 4 per cent. ammonium oxalate and neutral formalin or in formalin saturated with ammonium oxalate in the cold. Phosphates

and carbonates of calcium do not react by this method, according to its author, but Freudenberg (1926) maintained that the prolonged action of oxalate could cause these insoluble salts to react. Lison cautions against too strict an interpretation of the localization of soluble calcium by Rabl's method.

Ionized or Ionizable Forms. Calcium in these forms is identifiable by two methods whose specificity is high. In the first, the section is placed in 40 per cent. alcohol and treated with a single drop of 3 per cent. sulphuric acid. A coverslip is then applied and the section observed under the microscope. In a short while coarse crystals of gypsum ($CaSO_4.2H_2O$) are formed. The localization of the crystals is broadly over the area in which the calcium was previously situated, but definition on the cytochemical scale is impossible. Lillie (1954, p. 266) recommends as an alternative that 10 per cent. oxalic acid should be drawn under the coverslip by means of blotting-paper applied to the opposite edge. Crystals of calcium oxalate which form are easily recognizable, but localization is no more accurate by this than by the gypsum method. Turchini (1924) employed a solution of iodic acid in gelatine, which he applied to sections with the idea of forming insoluble calcium iodate, diffusion being kept to a minimum by the presence of gelatine. This process was designed for, and used upon, micro-incinerated specimens and it seems that it is not applicable to fresh or fixed tissues.

Masked Calcium. Calcium in this form is demonstrable by micro-incineration, applied either to fresh frozen sections, or to sections of fixed tissue or, preferably, to freeze-dried material. It is not proposed to deal with the technique in this book.

Insoluble Forms. The methods described below are principally applicable to insoluble deposits of calcium phosphate and carbonate, though some have been employed for the other forms. A method depending on the formation of a blue calcium lake was *Crétin's gallic acid-metaldehyde method* (Crétin, 1922, 1923, 1924) and, according to Lison (1936a), this had a high degree of sensitivity. Other metals were stated to give distinguishable colours with the Crétin reagent (barium, cadmium and strontium bluish-green ; iron dark brown ; and magnesium, zinc, lead and silicon various shades of yellow). Lillie (1954) continues to give details of the method but states that he has not, in fact, tried it. Bunting (1951), in a paper on the histochemical analysis of mineral deposits, found the Crétin reaction to be useless, and I agree entirely with this view. Gomori (1952, p. 33) regarded the Crétin test as too capricious to be dependable and Lison (1953, p. 162) no longer recommends it.

A second method making use of the formation of a calcium lake is the *Roehl and Leutert hæmatoxylin test*. Sections are treated for a few

minutes in a concentrated alcoholic solution of either hæmatoxylin or hæmatein, and deposits of calcium are coloured bluish-black. This method we customarily employ in principle when staining sections with Ehrlich's hæmatoxylin and eosin. Its specificity is obviously very low. It serves primarily to draw attention to the presence of calcium deposits but may be used more critically for the demonstration of these if a control section is stained identically after brief treatment (1–2 minutes) with dilute acetic (5 per cent.) or nitric (0·25 per cent.) acids. The blue-black calcium deposits of the test section will be absent in the case of the control. This reaction must not be used for critical histochemistry.

A third group of methods depending on the formation of calcium lakes employs various compounds related to alizarin. *Alizarin-purpurin methods.* The dyes used are all derivatives of anthraquinone. Grandis and Mainini (1900, 1902) used purpurin as a saturated alcoholic solution applied to alcohol-fixed frozen sections. Crétin (1924) and Cameron (1930) used alizarin sulphonic acid in the form of its sodium salt, Bourne (1943) used either this salt or 1 : 2 : 5 : 8-tetrahydroxy-anthraquinone for staining the calcium salts of bone *in vivo*. Langeron's (1934) method for staining calcium deposits in paraffin sections employed sodium alizarin sulphonate also. The early alizarin-purpurin methods possessed a low degree of sensitivity for calcium and their specificity was not high. Aluminium salts may give brilliant scarlet red lakes which are more satisfactory than the calcium lakes, often described as red but usually appearing in various shades of orange. Chromium, barium, strontium and iron also give coloured lakes with various anthraquinone derivatives, those of the last-mentioned being deep violet-black in colour.

A more sensitive technique using alizarin red S was evolved by Dahl (1952), who stressed the importance of pH in the performance of the test. Table 51, below, summarizes his findings in respect of the alizarin complexes of various elements.

The sensitivity of his alizarin method was stated by Dahl to be greater when used histochemically than in spot tests. With most inorganic methods it is my impression that the opposite is the case. For those who wish to try it this method is given in Appendix 24, p. 932.

In recent years other lake-forming, or chelating, compounds have been brought into histochemical use for demonstrating calcium deposits. Kaufman and Adams (1954) used a saturated solution of murexide (ammonium purpurate) in 0·1 N-KCN, and McGee-Russell (1955) described the use of the anthraquinone dye Nuclear fast red. This he proposed to call calcium red because of the ambiguity attached to the former name on account of the German equivalent, *Kernechtrot*, which is used for two very different classes of dye.

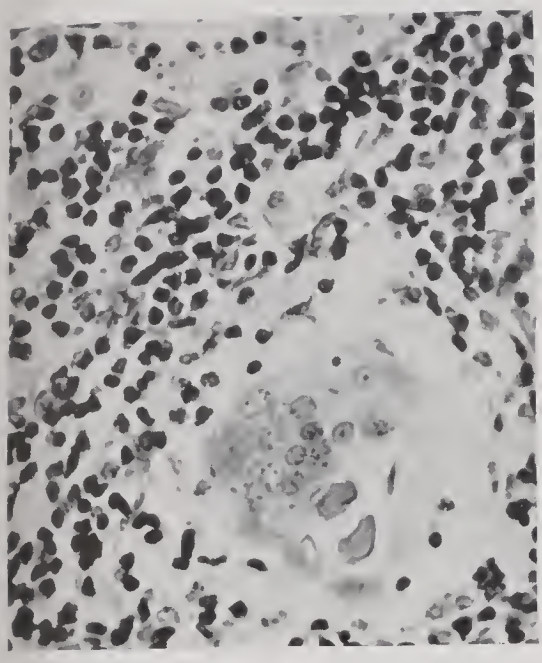


Fig. 209. As Fig. 211. Positive test for iron given by the outer layers of crystals present in one of the giant cells. Perls' reaction, neutral red. × 370.

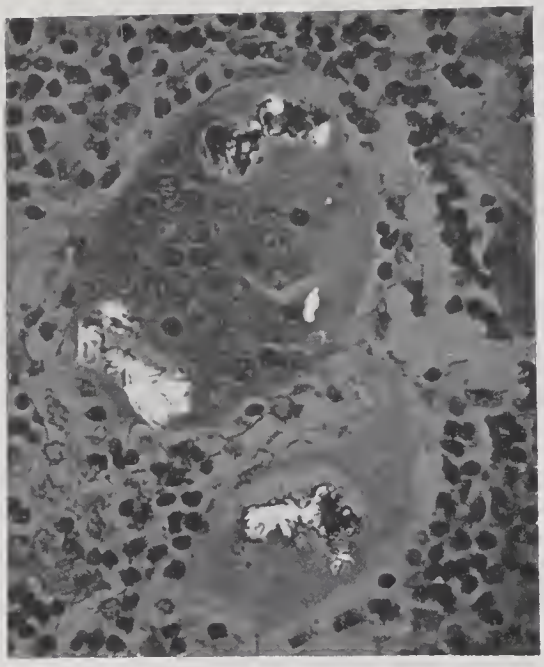


Fig. 210. As Fig. 211. To show birefringence of the crystals (prisms half crossed). × 370.

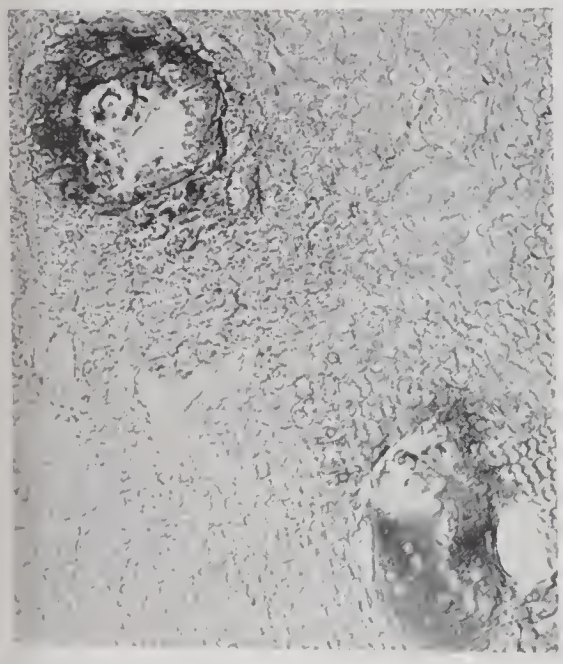


Fig. 211. Paraffin section (5·5 μ) of a human lymph node containing large numbers of foreign-body giant cells in most of which crystalline material was visible. To show localization of inorganic phosphate. Modified Serra method. × 125.

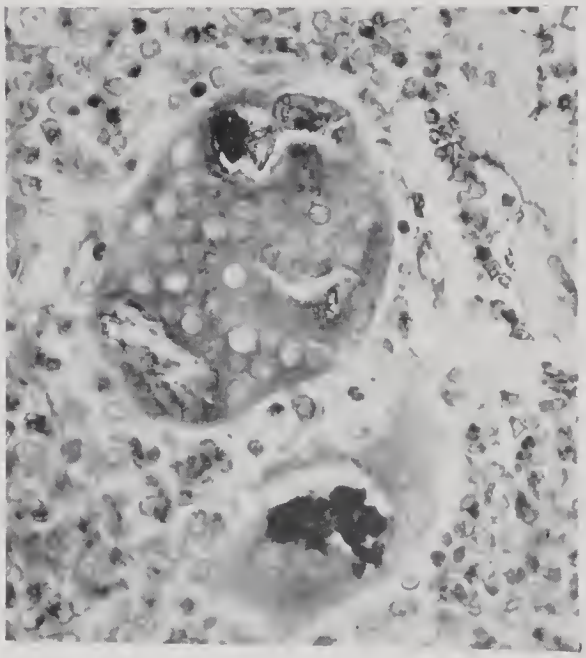


Fig. 212. As Fig. 211. Strong staining of the outer layers of the crystals with a phthalocyanin dye. Alcian blue, neutral red. × 370.

[*To face p.* 688.

FIG. 213. *Anodonta cygnea* (fresh water mussel). Gills. In each bar is a bright blue-stained mineral deposit containing a large amount of calcium. Copper phthalocyanin method. × 130.

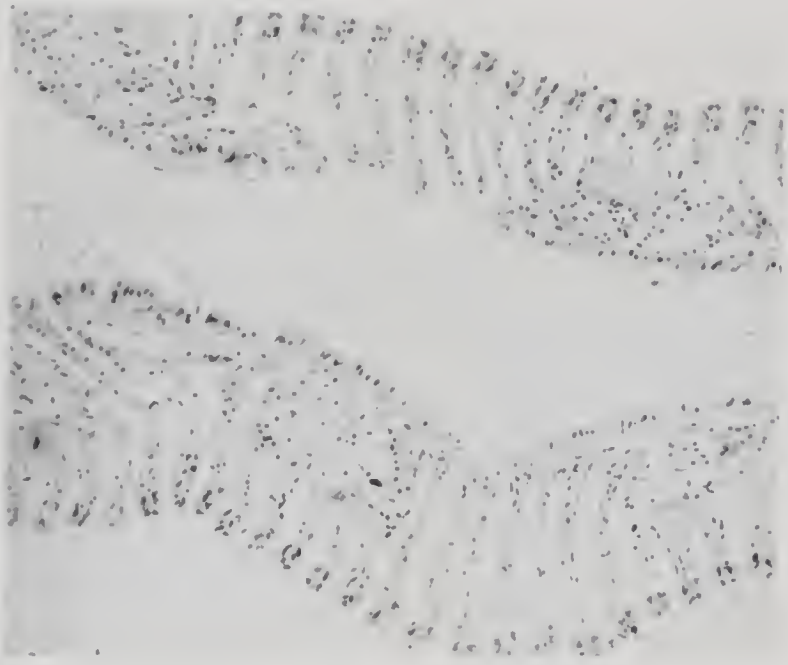


FIG. 214. As Fig. 213. Control section pretreated with 1 per cent. acid. Calcium deposits dissolved out. Copper phthalocyanin—neutral red. × 130.

FIG. 215. Human kidney. Nephrocalcinosis. Shows large amorphous calcium phosphate deposit, stained bright green. Mercury—naphthochrome green. × 130.

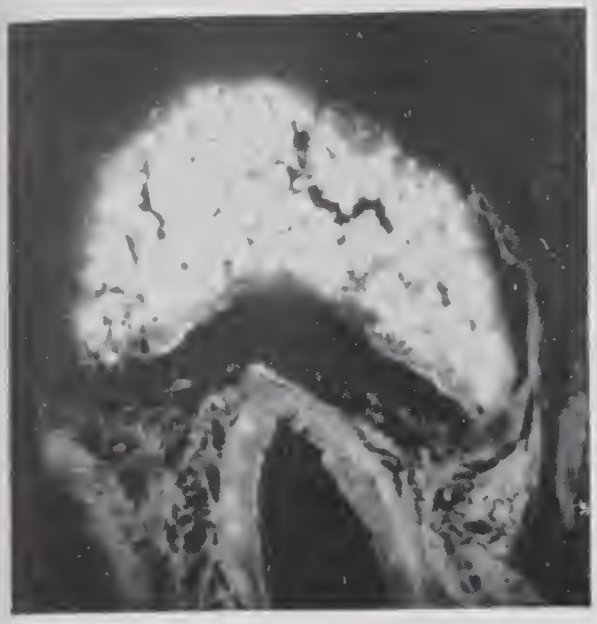


Fig. 216. Rat bronchus. Calcified cartilage shows bright whitish-green fluorescence. Morin method. × 110.

Fig. 217. Human gall bladder. Calcium deposits in fibrosed wall of the organ. Shown as brightly fluorescent areas. Morin method. × 100.

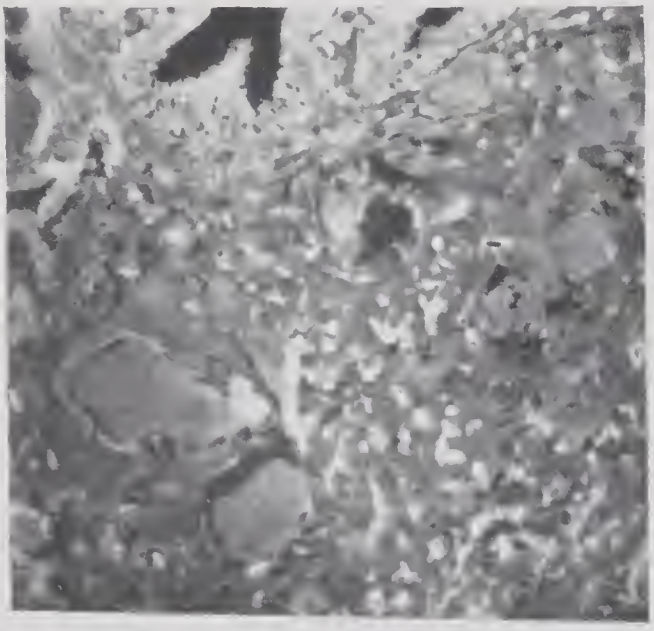


Fig. 219. Rat lung. Deposits of aluminium hydroxides shown as fluorescent spots. Morin method. × 100.

TABLE 51

Solubility of Alizarin Complexes

Salt	pH range (max. ppt.)	Colour
$CaCl_2$ . .	4·28–6·75	Reddish-orange
$FeCl_2$ . .	5·62–8·05	Deep purple
$Fe(NO_3)_3$ . .	5·73–6·25	Purplish-black
$Ba(C_2H_3O_2)_2$.	4·08–8·05	Reddish-orange-magenta
$SrCO_3$ . .	4·5 –8·05	Reddish-orange-magenta
$Be(NO_3)_2$ . .	5·73–8·05	Magenta
$CdCl_2$ . .	5·11–5·78	Reddish-orange or magenta
$La(C_2H_3O_2)_3$.	5·73–8·05	Cherry to light purple
$Pb(C_2H_3O_2)_2$.	4·08–4·5	Reddish-orange to purple
$HO_2(NO_3)_2$.	4·5 –5·62	Purple

The calcium red method (Appendix 24, p. 933) works very well in the case of many invertebrate tissues, but, in my hands, it is less reliable for calcium deposits in mammalian tissues where diffuse staining of the tissues usually results. Since the calcium-calcium red lake is water-soluble the shortest possible staining time should be employed.

Many members of the phthalocyanin class of dyes will stain calcium deposits (Pearse, 1955), and of these Durazol fast blue 8G and Alcian blue 8GS (I.C.I. Ltd.) are particularly useful. The former was employed to stain calcium in the gill plates of *Anodonta cygnea*, illustrated in Figs. 213 and 214, p. 688. Because of the effect on lake formation of the different physical states in which calcium occurs in the tissues it is often worth while to use a battery of phthalocyanins or a water-soluble followed by a spirit-soluble one (see Appendix 24, p. 933).

Kaufman and Adams refer to the use of Eriochrome Black T as a stain for tissue calcium. This dye forms complexes with Ca and Mg, which differ in colour from the tissues stained by the dye, and the nature of these complexes has been described by Young and Sweet (1955). I have had little success with this method, however, and cannot recommend it.

The dinaphthylphenylmethane dye Naphthochrome green B or G, which was used by Denz (1949) to stain deposits of beryllium (*q.v.*) in the tissues, forms a reddish-brown complex with calcium salts. In mercury-fixed tissues, however, calcium deposits are stained green. These are easily distinguishable from those of beryllium (pale apple green) or aluminium (rich dark green) by the fact that they are removed, before or after staining, by treatment with dilute acids. I consider

that after mercury fixation Denz's method (using 0·5 per cent. aqueous Naphthochrome green at pH ± 7·2 instead of the original pH 5·0), merits serious consideration as a routine method for demonstrating calcium salts. In my hands it is one of the best of the chelation techniques, having a coloured product visible in ordinary light (see Fig. 215, p. 688).

Fluorescence Method for Calcium and Other Metals

According to Feigl (1954) alcoholic solutions of 3,5,7,2′,4′-pentahydroxyflavanol (morin) react with salts of aluminium, beryllium, indium, gallium, thorium, scandium and zirconium to give fluorescent complexes or adsorption compounds. Aluminium and beryllium (*q.v.*) are the only two metals in this list whose salts are at all likely to be found as foreign substances in mammalian tissues. I have observed, however, that calcium deposits as well as calcium in bone and cartilage give an intense whitish-green fluorescence when treated with morin in acetic-alcohol solution. This observation forms the basis of the fluorescent reaction for calcium described in Appendix 24, p. 935.

Distinction between the fluorescence of the morin-calcium complex and those of other metals might possibly be made on the difference in colour (fluorescence spectrum). The aluminium complex, for instance, gives a much greener fluorescence than does the calcium lake (see p. 693). The morin reaction in calcified cartilage from the bronchial tree of the rat is shown in Fig. 216, p. 688, and in mixed amorphous and crystalline calcium phosphate deposits in a thickened gall-bladder wall in Fig. 217.

The sensitivity of this method, histochemically, is higher than that of any of the visible light chelation methods, so that very small deposits of calcium phosphate or carbonate can easily be distinguished. Tissues containing calcium salts in non-particulate form also give the characteristic fluorescence.

Substitution Methods for Calcium. It is customary in routine histology to use the *silver method of von Kóssa* (1901) for the demonstration of calcium deposits. This depends on the presence of phosphate and carbonate and not on the presence of calcium. In animal tissues, however, insoluble phosphates and carbonates are nearly always those of calcium and the test is usually regarded as sufficiently specific for this element. The principle of the method is the conversion of either salt into its silver equivalent and the subsequent reduction of this by means of sunlight or ultra-violet light, or by means of a strong reducing agent, to black metallic silver. None of the many substances which reduce silver nitrate to metallic silver is likely to cause confusion except perhaps uric acid or urates. Gomori (1951) recommended the previous removal of these by treating sections with saturated aqueous lithium carbonate.

TABLE 52

In Vitro Tests on Reagents for Metals

METAL	Rubeanic acid	*o*-Tolidine	Benzidine	Rhodizonate	Diethyldithiocarbamate	Dithizone	DMABR *	Naphthochrome green	Acid solochrome cyanine	Solochrome azurine	Magneson	Titan yellow	SALT
Be^{2}								**Green**	**Purple**				$BeCl_2$
Mg^{2}											**Blue**	**Red**	$MgCl_2$
Al^{3}				Yellow		Orange		**Deep green**	**Rose-pink**	**Purple**			$Al(OH)_3$
Ca^{3}					Gold	Grey-green				Purple			$CaCl_2$
V^{5}				Yellow				Grey	Pink	Pink			V_2O_5
Mn^{2}					Brown	Light brown	+			Red			$Mn(NO_3)_2$
Fe^{2}	Yellow			**Blue-black**	**Black**	Light brown	+	Green		Blue			$FeSO_4$
Fe^{3}		Blue	Dark blue	**Blue-black**	**Black**	Pink	(+)	Green		Blue			$Fe_2(SO_4)_3$
Co^{2}	Yellow				Lime-green	Pink	+	Blue-grey		Purple			$Co(NO_3)_2$
Ni^{2}	**Blue**				Yellow	Brown-red		Green		Reddish-blue			$NiCl_2$
Cu^{1}	**Olive**	Light blue			Yellow-brown	Brown	++ **Red**	Green		Blue			$CuCl_2$†
Cu^{2}	**Olive**	**Dark blue**	**Blue-black**	Yellow	Yellow-brown	Brown	+ Brown	Green		Blue			$CuSo_4$
Zn^{2}						**Deep red**		Grey-green		Purple			$ZnSo_4$
Sr^{2}				**Red**						Pale blue			$SrCl_2$
Bn^{2}					Yellow	Pink	+			Orange			$SnCl_2$‡
Ba^{2}				**Red**		Brown		Red		Orange			$Ba(OH)_2$
Hg^{2}						Red	++ Purple	**Green**		Purple			$HgCl_2$
Pb^{2}	Yellow			**Purple**			++ Purple	Black					$Pb(NO_3)_2$
Bi^{3}						Brown-red				Orange			$BiCl_3$

* Dimethylaminobenzylidene rhodanine.
† Dissolved in NH_4OH and evaporated to dryness.
‡ Dissolved in 5 N HCl.

The other substitution methods for calcium salts are not used for the demonstration of deposits. The cobalt and yellow ammonium sulphide method, which is used for revealing calcium orthophosphate in the alkaline phosphatase technique (Chapter XIV), gives poor results on older calcium deposits. The method of Macallum, which involved conversion of the deposits to sulphate followed by treatment successively with lead acetate and yellow $(NH_4)_2S$, was shown by Lison (1936a) to be completely useless.

Methods for Various Metals

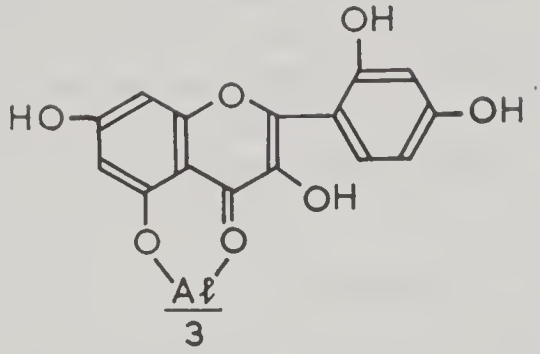
In the first edition of this book only the briefest reference was made to some of the methods which now appear in this section. My original objection to most of them was that it was necessary to introduce the metal or its salts into the tissues in relatively enormous quantities before a positive result could be obtained, and trace elements, present in physiological amounts, could not usually be demonstrated. In the last few years the position has been altered by various technical improvements, and I have therefore thought it worth while to consider in this chapter a number of the improved methods as well as a few of the older ones which still find some use in research studies. Additionally, a few methods are considered which have not yet found employment in histochemistry but which, if successfully adapted, may be expected to yield helpful information.

Methods for Aluminium

This element is not normally present in mammalian tissues, but powdered aluminium has been introduced into the lungs as a preventative for silicosis (Denny, Robson and Irwin, 1937). After such therapy it is present as a mixture of metal and metal hydroxide. Hydroxides of aluminium are known to form adsorption complexes with a variety of compounds, alizarin, quinalizarin, morin and aluminon among them, and the latter (ammonium aurine tricarboxylic acid) has been described by Hammett and Sottery (1925) as a specific reagent for aluminium. Denny *et al.* (1939) first described the histochemical use of the *aluminon method*, and a modification of this was used by King, Harrison, Mohanty and Nagelschmidt (1955) for the demonstration of aluminium (hydroxide) in rat lung. The reagent is used in solution as the ammonium salt and differentiation is performed with ammonium carbonate. Lakes formed by the combination of aurine tricarboxylic acid and metals other than aluminium or beryllium are dissolved by ammonium ions at pH 7·2, and therefore by ammonium carbonate. In the absence of excess dye the beryllium lake is unstable so that the method becomes specific for aluminium salts. This method works reasonably well (Fig. 218, p. 696), though it sometimes fails inexplic-

ably when aluminium is present in the tissues in large amounts by chemical assay.

A second method which may be used to demonstrate aluminium hydroxide histochemically is the *morin method* already described for calcium. According to Feigl (1954) an inner complex salt is formed



and this gives an intense greenish fluorescence. The identification of aluminium dust in lungs (Fig. 219, p. 688) is more easily made by this method than by the aluminon method described above and distinction from calcium salts (by acid pretreatment) is also easy.

The *naphthochrome green method* (at acid or alkaline pH levels) gives a rich green lake with aluminium hydroxide (Fig. 220). When, as with aluminium dust inflation experiments, the nature of the metal introduced into the tissues is known, this method is far more satisfactory than the more specific aluminon method.

A fourth method for aluminium salts, whose sensitivity and specificity appears to be high, is the *Solochrome blue-black R method.* In acid alcoholic solution this dye forms reddish chelates with salts of aluminium and calcium. The latter are first dissolved and then reprecipitated as the calcium-solochrome lake, which is insoluble in dilute acids. The new precipitate is diffuse, however, and in place of, say, a crystal of calcium phosphate measuring $2 \times 2\ \mu$ there is now a circular patch of amorphous granular deposit 100–200 μ in diameter. Moreover, while these granules do not fluoresce, the Solochrome-aluminium chelate fluoresces brilliantly in shades varying from yellowish-orange to strawberry-pink Plate IXA, p. 681). These stand out clearly on a pale blue background. Other Solochrome dyes give coloured, non-fluorescent complexes with aluminium hydroxide. Methods employing these dyes are given in Appendix 24, pp. 935 and 936, and they are illustrated in Plates IXB and IXC, p. 681.

Methods for Arsenates and Arsenites

The method most commonly used for the histochemical demonstration of As^{+++} and As^{++++} is that of Castel (1936), in which both arsenites and arsenates are precipitated as their cupric salts by fixing the tissues in a medium containing $CuSO_4$. The resulting green granular precipi-

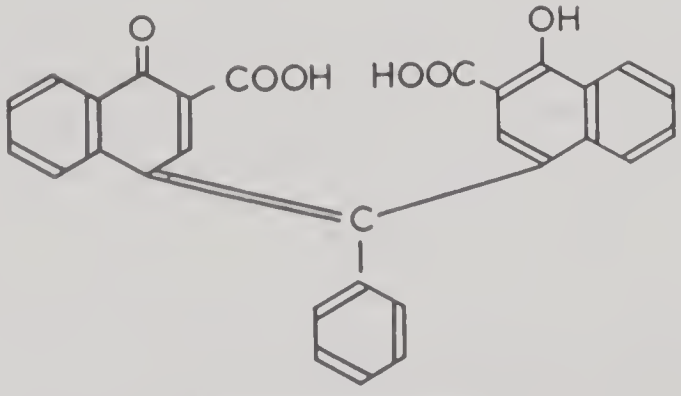
tate is visible microscopically. I have no experience of this method, but alternatives would seem to be worth trying. The method of Bettendorf (1870) is mentioned by Feigl (1954) as highly specific. It is based on the principle that ter- and quinquevalent arsenic compounds are reduced to the metal by warming with stannous chloride in 35 per cent. HCl. Arsenic appears as a brownish-black precipitate. A test for As^{+++} described by this same author depends on the development of a red colour when arsenites are warmed with 0·5 per cent. *n*-ethyl-*o*-hydroquinoline (kairine) and 1 per cent. ferric chloride in strong HCl solution. Arsenates are previously reduced with hydroxylamine sulphate.

Method for Barium and Strontium

The sodium rhodizonate test for these two metals was described by Feigl (1924) and adapted by Waterhouse (1951a and b) for histochemical use. Intensely red or reddish-brown compounds are formed; calcium salts do not react and distinction between barium and strontium is made by treatment with potassium chromate. If this is applied before the rhodizonate it prevents staining of barium salts, and if afterwards, their colour is discharged. Gomori (1952) questions the specificity of this method, remarking that Hg and Pb also give red rhodizonates. This is true (although from neutral solutions Pb gives a violet salt), but distinction of this sort is unlikely to be required histochemically. What is more usually called for is the localization of some already chemically determined metal. In practice deposits of barium sulphate, in tissues or on filter paper, give an almost black colour after rhodizonate treatment (Appendix 24, p. 938), and this is converted to a fine scarlet red by treatment with strong mineral acids. Ferric and ferrous salts give a black colour with rhodizonate (see Table 52, p. 691) and some forms of tissue iron react in this way.

Methods for Beryllium

The only histochemical method so far employed for salts of



Naphthochrome green B.

beryllium is that of Denz (1949) using *Naphthochrome green*, already described in this chapter as a reagent for calcium and aluminium salts (*q.v.*). This method has been used successfully by several authors. Firket (1953), for instance, demonstrated Be in cells in tissue culture, and Cheng (1956) localized the metal in the Kupfer cells in the liver of experimental berylliosis. The specificity of the test for beryllium depends on exclusion of Ca and Al salts, and this is done by performing the reaction at pH 5·0 when only very high concentrations of aluminium will react, and calcium salts will dissolve.

Many types of beryllium deposits appear dark blue to almost black in hæmatoxylin and eosin preparations. This is due to the formation of a hæmatoxylin lake which cannot be regarded as a specific test although it may draw attention to the presence of the deposits.

Since many beryllium salts fluoresce it is always worthwhile to examine an unstained section in ultra-violet light. The deposits often emit a characteristic bluish-white fluorescence, and this can be quenched by several different chelators such as alkaline quinalizarin, for instance. The *morin* (*fluorescent*) *method* for beryllium salts was described by Zermatten (1933), and according to Feigl (1954) a yellowish-green fluorescent compound is formed when alcohol or acetone solutions of morin come into contact with $Be(OH)_2$. If the deposits are autofluorescent this method is not applicable although a slight increase in fluorescence can be observed after performing the morin reaction in such cases.

A third method for beryllium is the *alkaline quinalizarin method* (Appendix 24, p. 938). This colours the deposits a deep purple (Fig. 221, p. 697), which changes to brown in acid solution. Distinction from the neodymium, praseodymium, cerium, lathanum, thorium and zirconium lakes of quinalizarin is unlikely to be required in histochemical practice. The magnesium lake is blue, but can be distinguished from the beryllium lake by its stability to bromine in the presence of sodium hydroxide. This destroys the colour of the beryllium lake, while bromine in the presence of ammonia destroys only the magnesium lake. Beryllium deposits can usually be rapidly and specifically demonstrated with 0·5 per cent. aqueous *Solochrome azurine*, which stains them deep blue (Plate IXD, p. 681). Differentiation from Ca and Al is easy (see Appendix 24, p. 938).

Method for Bismuth

This metal is found in the tissues for an indefinite period, after its injection for therapeutic purposes, in the form of brown or black granules. These are identifiable as bismuth by the Komaya-Christeller method (Komaya, 1925 ; Christeller, 1926) or by a modification described by Castel (1935). The latter's brucine method was further

modified by Wachstein and Zak (1946), and their method appears in Appendix 24, p. 939. I have no experience of its use.

Methods for Cobalt and Nickel

Cobalt salts can be demonstrated in quite small amounts in the tissues by conversion to the brown sulphide. This is done in many histochemical enzyme reactions where the precipitate contains cobalt. Other chemical methods for this metal (α-nitroso-β-naphthol, rubeanic acid, ammonium thiocyanate) have been used in histochemistry by Voigt, Bog, and Wolf (1954). They found that only the sulphide method gave accurate localization in the tissues.

Nickel salts have been shown by Wells (1956) to have a considerable affinity for certain keratins, in fixed or unfixed state. These keratins can take up in a few hours, from solutions containing 1 p.p.m. of nickel, a sufficient amount of the metal to give a mauve to bluish colour with rubeanic acid (dithiooxamide). The dimethylglyoxime method can also be used to demonstrate nickel salts taken up by keratin in this way.

Methods for Copper

Although copper is present in traces in most vertebrate tissues the amount is too small to be detected histochemically by any of the existing methods. Increased amounts are said to be present in fœtal and cirrhotic livers (Okamoto and Utamura, 1938) and in the liver of hepato-lenticular degeneration relatively enormous amounts (up to 30 mg./100 grams) occur. Such figures are at least ten times higher than the highest figures for normal tissues. Enzymes of the mono- and polyphenol oxidase classes are copper proteins, and the tyrosinase of the wall of the ink sac in the octopus and squid is especially rich in copper. The respiratory proteins of *Mollusca* and *Arthropoda* contain copper in place of the iron of hæmoglobin, and in some marine species the total copper content of the organism reaches a high level. Echave (1941) includes a number of estimations on such materials in his monograph on the biological use of copper.

Most of the older methods for copper used in histochemistry lack both specificity and sensitivity and must be abandoned. Such methods include the hæmatoxylin-lake method of Mallory and Parker (1939), and the same authors' methylene blue technique. A dilute hæmatoxylin method was used by Mendel and Bradley (1905) in their studies on the distribution of copper and zinc in the liver of the gastropod *Sycotypus canaliculatus* (Figshell) collected from Long Island Sound. They used $(NH_4)_2S$, and a potassium ferrocyanide method, with equally good results. Lison (1953) condemned most of these methods, but continued to support the use of the potassium ferrocyanide test as a specific

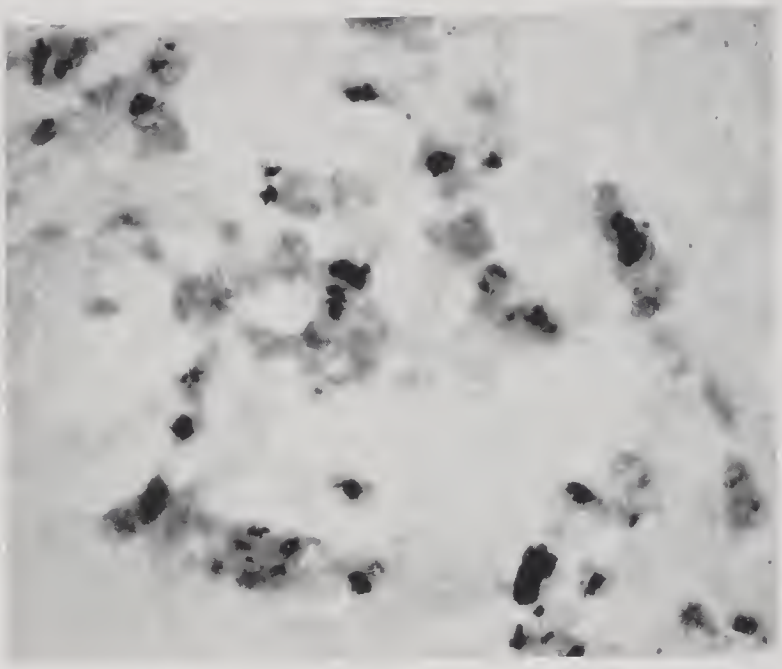


FIG. 218. Rat lung. After insufflation of metallic aluminium dust. Aluminon method. × 510.

FIG. 220. Rat lung. As Figs. 218 and 219. Aluminium deposits stained bright green. Naphthoehrome green B. × 510.

[*To face p.* 696.

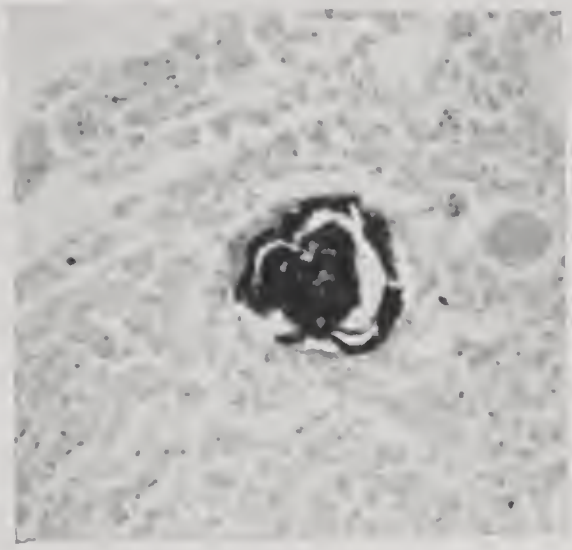


FIG. 221. Human lung. Case of beryllosis. A large deposit of beryllium stained deep purple. Alkaline quinalizarin method. × 130.

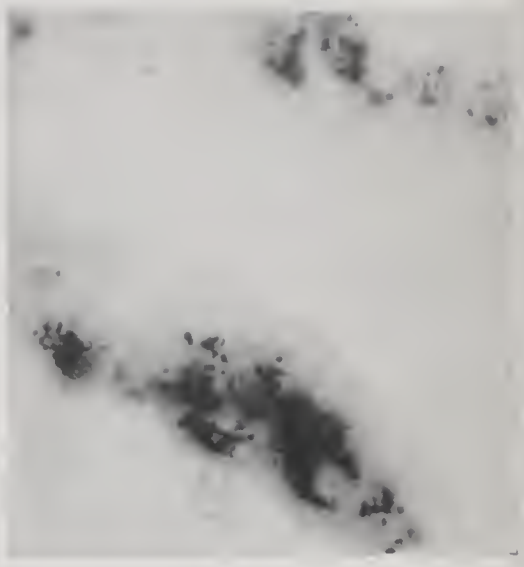


FIG. 222. *Triton rubicundus* (whelk). Copper-containing pigment granules in macrophages. Rubeanic acid method. × 510.

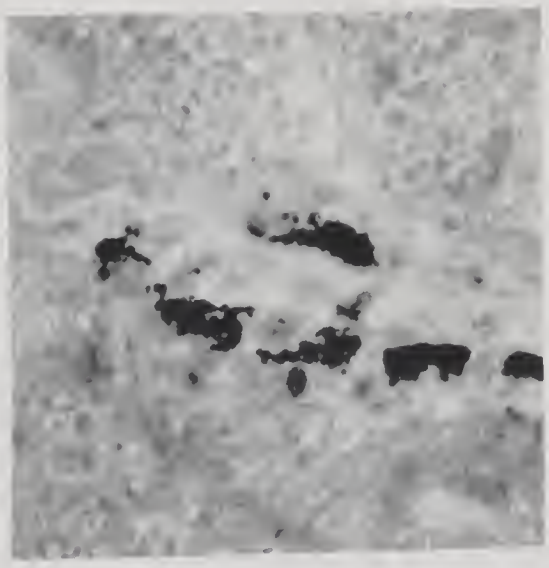


FIG. 223. As Fig. 222. Dimethylaminobenzylidene rhodanine. ×510.

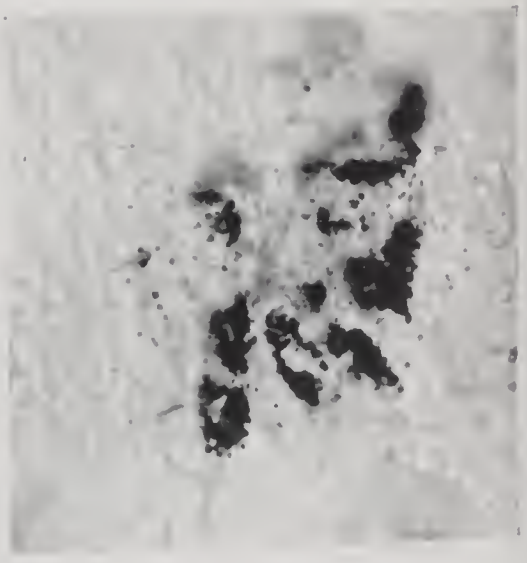
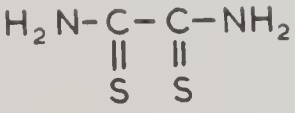
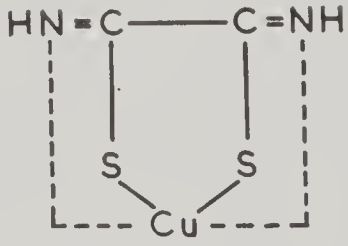


FIG. 224. As Figs. 222 and 223. Diethyldithiocarbamate. ×510.

reaction. Its sensitivity is certainly not high enough to demonstrate physiological or pathological levels of copper in mammalian tissues. As their final method Mendel and Bradley used a concentrated solution of hydrobromic acid containing free bromine, which gives a violet colour with copper salts. They found the reaction " brilliant and very delicate," but the coloured compound was observed to diffuse rapidly through the tissues and thereafter to fade. Lison regarded this reaction as highly specific. Although its sensitivity in spot tests (0·15λ Cu) is high enough, Feigl (1954) points out that Ag, Hg, Fe and Cl interfere with the reaction. In histochemical practice only artificially high levels of Cu^{++} can be demonstrated in mammalian tissues.

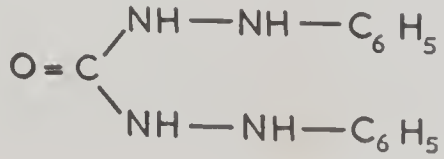
There remain to be discussed seven more modern methods. The first, and probably the most important of these methods in mammalian histochemistry, employs *rubeanic acid* (*dithiooxamide*). This compound has already been mentioned as giving a coloured chelate compound with nickel salts. It was first used successfully for the histochemical localization of copper by Okamoto and his associates (1938, 1939), and it gives a deep greenish-black colour with salts of this metal. Feigl (1954) regards this as due to formation of an inner complex salt of the di-imido form of rubeanic acid.





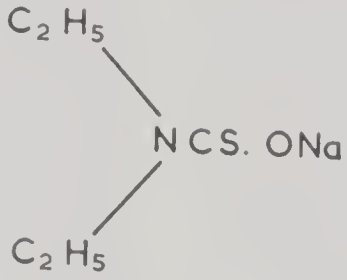
In the alkaline solution used in the test only copper, cobalt and nickel salts give a positive reaction, but the colours (greenish-black, yellowish-brown and mauve) are easily distinguishable. Moreover, if the medium contains ethanol and acetate ions Co and Ni rubeanates are soluble. Fig. 222 shows the result in the case of the copper pigment of *Triton rubicundus*.

Another method was described at the same time as the above by Okamoto *et al.* which made use of *p-dimethylaminobenzylidene rhodanine* (Fig. 223). This compound reacts to form a reddish-brown precipitate. It is more usually employed for the demonstration of mercury and silver (*q.v.*). The third method suggested by Okamoto *et al.*, which does not yield permanent preparations, is the *diphenylcarbazide* method. This compound has the following structure :



and it reacts with copper salts in neutral alcoholic solutions to give a reddish-violet colour. In strongly acid solutions only hexavalent chromium and molybdenum react.

Waterhouse (1945a, b) made a number of studies on the distribution of copper in the sheep blow-fly *Lucilia cuprina*, and for these he employed a fourth compound, *sodium diethyldithiocarbamate* (Fig. 224), which has the structure.

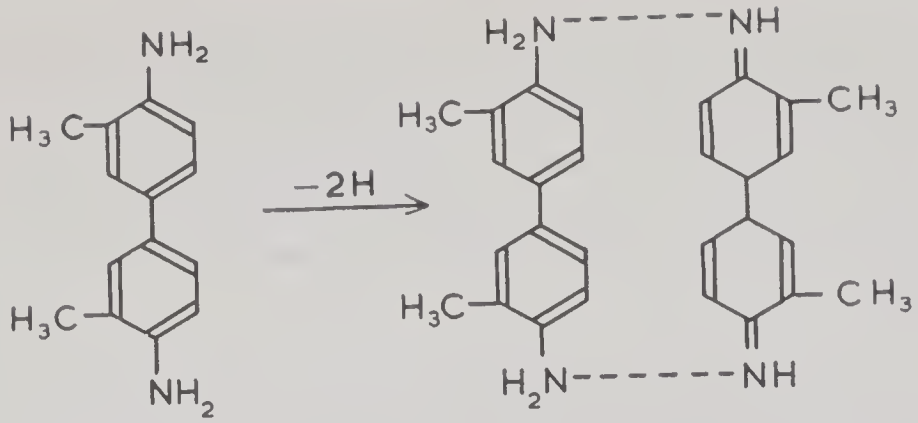


It was originally used as a copper reagent in the form of its zinc salt by Clarke and Hermance (1937). Whichever salt is used the yellowish-brown cuprous derivative is precipitated. The *diphenylthiocarbazone* method gives coloured precipitates with a number of metals, including Ag, Cu, Ni, Cd and Zn. In neutral or ammoniacal solution it gives a yellowish-brown precipitate with copper salts, but it has not found much employment in mammalian histochemistry.

Cupric ions form an inner complex salt with *dithizone* (see section on Zinc, below), and this is yellowish-brown in colour. The sensitivity of this method is not high enough to give a positive result in most mammalian tissues, but it will do so in the case of certain marine invertebrates where the concentration of copper per unit of tissue is much higher and where, in fact, the metal is found only in pigmented granules in phagocytic cells so that the histochemical concentration is even higher.

The final group of methods may be described as *oxidation catalyst reactions*. Copper salts are known to react with *benzidine* in the presence of alkali cyanides to give benzidine blue, and Feigl (1954) recommended the substitution of *o-tolidine* for benzidine in order to increase the sensitivity of the reaction. Both these methods depend on the fact that the oxidation potential of Cu^{++} salts is raised when Cu^{+} ions are removed by the formation of insoluble cyanides. Like benzidine blue (see p. 518), tolidine blue is an unstable compound easily reduced to a colourless product or oxidized to a brown one.

Interference with the specificity of the tolidine reaction for copper might be expected to come from salts of silver, mercury, iron, thallium, cerium and gold, but, in histochemical practice, iron is the only one likely to be of importance (see Table 52, p. 691). Hæmoglobin, hæmosiderin, and other forms of naturally-occurring iron in mammalian tissues, however, give entirely negative results when tested by this



method. When used for the demonstration of copper in invertebrate tissues the tolidine reaction shows a higher degree of sensitivity than any of the other methods listed above. Copper-containing pigments in the perivascular phagocytes in the hepatopancreas of *Triton rubicundus* are well shown by its use, but in mammalian tissues I have not yet succeeded in obtaining a positive result, even in material known to contain copper (natural) in excessive amounts.

Unreactive Copper

It is thought that Cu^{++} or Cu^{+} may be present in some tissues in an unreactive form from which they must first be released before they can react. Gomori (1952) recommended the use of hydrogen peroxide for this purpose, and oxidizing agents of various types are commonly used. They are not altogether effective and Uzman (1956) has used concentrated HCl, applied to sections in the form of its vapour (see Appendix 24, p. 940). Chelators of various types may possibly release bound copper, as BAL (p. 104) does *in vivo*, but even if successful their chief use would be to provide negative controls for methods depending on the formation of coloured chelates.

The Applied Histochemistry of Copper

The majority of workers using mammalian tissues have shown a strong preference for the rubeanic acid method, and it is the only one with which I have been able to demonstrate copper in the liver and kidney in Kinnier-Wilson's disease (hepato-lenticular degeneration). Brand and Takats (1951) were able to obtain a positive result also in the characteristic Kayser-Fleischer rings in Descemet's membrane (hornhautringes). Working with tissues from a case of this disorder Green (1955), however, obtained positive results equally well by the Mallory (hæmatoxylin), rubeanic acid, diphenylcarbazide and diethyldithiocarbamate methods. Using fresh as well as formalin-fixed tissues from a similar case, Uzman (1956) obtained excellent results with a modifica-

tion of the Okamoto-Utamura rubeanate method. He regarded the original method as deficient in two major respects as a histochemical reaction. First, he considered that the use of watery solutions might lead to diffusion of copper, and secondly, he believed that failure to use copper-free materials could cause serious artifacts to occur. Unfortunately Uzman did not consider any of the other available methods for copper.

Discounting for the moment the *o*-tolidine oxidation method, which has an *in vitro* sensitivity of 0·003γ Cu^{++}, histochemical comparison of the remaining methods suggests that rubeanic acid (sensitivity 0·006γ) is definitely superior to dithizone (0·1γ), diphenyldithiocarbamate (0·002γ), and dimethylaminobenzylidene rhodanine (0·6γ), for demonstrating naturally-occurring copper. In the case of tissue sections cut from blocks fixed in copper sulphate, and containing varying amounts of copper, I found these differences harder to detect. All three methods (dithizone excepted) appeared to become positive at much the same level of copper although the colour of the rubeanate was the strongest. In these artificially prepared tissues the sensitivity of the *o*-tolidine reactions was higher than that of any of the other methods.

In insect tissues the diethyldithiocarbamate method has been preferred to the rubeanic acid method by Waterhouse (1951a and b), who failed to obtain a positive result at all with the latter. It was preferred also by Poulson and Bowen (1952) for their studies on copper metabolism in *Drosophila* larvæ. According to Clarke and Hermance (1937) the sensitivity of zinc diethyldithiocarbamate for cuprous ions *in vitro* is 0·002γ, and it seems likely that copper in insect tissues is present in cuprous form rather than in the cupric form which is usual in mammalian tissues. If this is true it would account for the invariable failure of the dimethylaminobenzylidene rhodanine method in the latter. This compound reacts only with cuprous salts, or with cupric salts, in inorganic combinations, after their reduction by sulphurous acid.

Two new reagents for copper have recently been described. The first, bis-cyclohexanone oxalyldihydrazone (Peterson and Bollier, 1955), is used in alcoholic solution and is said to be twice as sensitive as diethyldithiocarbamate. The second method, described by Gehauf and Goldenson (1955), makes use of a dye called Zolon red. This forms red chelate compounds with silver and with cuprous salts.

Methods for Gold

Gold salts are sometimes introduced into the tissues therapeutically either in the form of radioactive or inert compounds. Two methods have been recommended for demonstration of the latter. The first is the method of Christeller (1927), which works well in the case of

inorganic gold but gives negative results where the metal is part of an organic molecule. This method depends in the reduction of the gold salts to purple of Cassius with stannous chloride in strong HCl, but in practice reduction to metallic gold (brown or black) also occurs. The degree of localization afforded by this method is not likely to be precise, as pointed out by Gérard and Cordier (1932), since solution of the original gold salt necessarily precedes the precipitation of purple of Cassius.

A second method was described by Elftman and Elftman (1945). It depends on the bleaching of all pigmented substances (except silver and gold) by means of the prolonged action of 30 per cent. hydrogen peroxide.

Although water-soluble Au^{+++} salts produce a purple colour with dimethylaminobenzylidene rhodanine this method does not seem to have achieved any histochemical success.

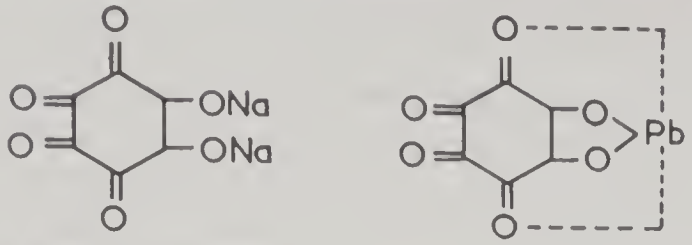
Methods for Lead

A number of dyes form coloured lakes with lead salts or $Pb(OH)_2$, among them hæmatoxylin, gallocyanin and carminic acid. Mallory's (1938) method employed a freshly prepared hæmatoxylin solution, and lead salts were said to stain bluish-grey. These lake methods are neither sensitive nor specific and their use is not recommended.

The most popular method for demonstrating Pb in tissues is the *chromate method* (Crétin, 1929 ; Frankenberger, 1921), in which the tissues are fixed in neutral potassium dichromate (Regaud) or, if already fixed, are treated with this mixture subsequently. Lead is found in the form of opaque yellow crystals.

Alternatively, *the sulphate method* is employed (Timm, 1936 ; Sieber, 1939). Tissues are fixed in absolute alcohol saturated with H_2S, which transforms lead salts into the brownish-black lead sulphide. Since this is insoluble in dilute and moderately strong acids, bones in which it has been deposited can be decalcified successfully. Lead chromate is soluble in acids and the chromate method cannot therefore be used for bone. Lison (1953) prefers the chromate to the sulphide method largely on the grounds that the former affords superior fixation. I have no experience of either. Lillie (1954), following up Molnar's (1952) suggestion to use sodium rhodizonate for the demonstration of the lead-precipitated reaction products of enzyme activity, has suggested this method for the demonstration of lead deposits in general. From the information given by Feigl (1954) it would appear that sodium rhodizonate is more likely to give positive histochemical results than dithizone or any other metal chelating agents, since it is capable of reacting even with the very slightly soluble PbS, $PbSO_4$ and $PbCrO_4$.

The type of chelate compound formed is illustrated below :



Sodium rhodizonate

In practice I have found that small concentrations of an insoluble lead salt ($PbSO_4$) in the tissues can readily be demonstrated by the rhodizonate method.

Methods for Magnesium

There are three histochemical methods for magnesium, two of which, the *titan yellow* and the *magneson* methods, are of high specificity. The third, the alkaline quinalizarin reaction, has already been mentioned as a method for beryllium (p. 695).

The titan yellow reaction was introduced into botanical histochemistry by Broda (1939), but it is equally applicable to invertebrate and mammalian tissues. A striking flame red colour is produced but, unfortunately, no method is known by which this can be stabilized and it fades within 24 hours. Magnesium-containing deposits in the mantle region of *Limnæa* are shown in Fig. 225, p. 704, stained by this method.

The magneson method depends on the affinity for magnesium salts of certain azo dyestuffs of which *p*-nitrobenzene-azo-1-naphthol is the best known. This dye is reddish in colour and it is adsorbed by magnesium salts without change in colour. When the medium is made strongly alkaline (pH 12) by the NaOH the colour changes to bright blue. This is also unstable in tissue due to the difficulty of maintaining so high a pH. Staining by this method gives precise and clear localization of magnesium deposits, as indicated by Fig. 226, p. 704. Both methods are given in detail in the Appendix to this chapter (p. 943).

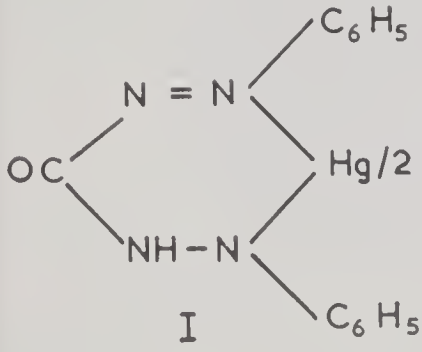
Methods for Mercury

Early histochemical methods for this metal depended on the conversion of its salts either to black HgS or to metallic Hg. The older *sulphide methods* in which H_2S was applied in the fixative have long been abandoned, the work of Lombardo (1906, 1908) having shown that H_2S, when added to the fixative, produces a whole spectrum of pigments from organic as well as inorganic precursors. The more modern sulphide methods employ treatment with H_2S after a variety of fixatives. They are not much employed although Lison (1953) regarded their sensitivity as high.

Reduction methods are still used and stannous chloride remains a popular agent for this purpose. It was used by Debenedetti (1925) and by Hand, Edwards and Calcy (1943) to reduce both mercurous and mercuric forms. The latter authors also used thioglycolic acid for reducing the mercurous form only.

Diphenylcarbazide and diphenylcarbazone methods were introduced to histochemical use by Brandino (1927), who used the first compound in alcoholic solution. His method, according to Lison (1953), showed only mineral compounds of mercury and consequently failed to reveal mercury in organic combination. The colour developed was violet.

Feigl (1954) indicates that the second of these two compounds forms an inner complex mercury salt with the constitution I. It is possible, however, that the constitution of the complex may be as shown in II.



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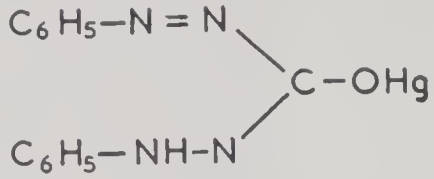


II

The specificity of the reaction for mercury depends on the pH. In neutral, or slightly acid, solutions other heavy metals (Cu, Fe, Co) react to give coloured compounds. After treatment of tissues with alcoholic mercuric nitrate, as in the phospholipid reaction of Okamoto *et al.* (1947) (see Chapter XI, p. 314), a very strong reddish-purple colour is produced in structures which retain mercury. These same tissues, however, fail to react by the dimethylaminobenzylidene rhodanine method, which *in vitro* has a sensitivity for salts of mercury not much below that of diphenylcarbazone.

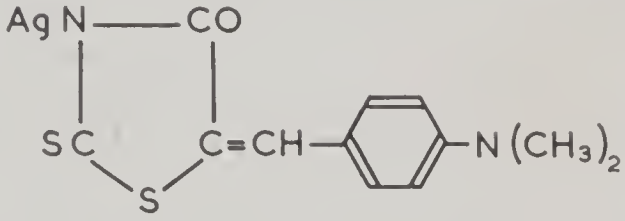
Methods for Silver

When silver salts are introduced into the tissues they are rapidly transformed into brown or black deposits of unknown constitution, presumably containing metallic silver bound to some protein carrier. Seen through the skin in cases of therapeutic argyrosis the deposits appear steel-blue. They have been identified histochemically mainly by their removal or decolorization with various reagents which do not affect melanin in the same period of time. A solution of 1 per cent. potassium ferricyanide in 20 per cent. sodium thiosulphate is recommended by Lison or, as an alternative, 1 or 2 hours' treatment with Lugol's solution followed by treatment with thiosulphate. Yellow

ammonium sulphide turns silver deposits into the black AgS, while potassium dichromate forms the brownish-red silver salt. These two salts are soluble in 5 per cent. aqueous KCN.

Two alternative methods were proposed by Okamoto, Utamura and Akagi (1939), neither of which is to be regarded as specific for silver. The first is the rubeanic acid method (see section on Copper, above) and the second the dimethylaminobenzylidene rhodanine method. Silver salts give a brown precipitate with neutral or alkaline solutions of rubeanic acid, but they will not react under the conditions given in Appendix 24, p. 940, where the method is used for detection of copper.

Rhodanine and similar compounds form insoluble silver salts selectively in acid solution, the silver taking the place of H in the cyclic imine (NH) group. At alkaline pH levels a number of metals form coloured insoluble rhodanates. The silver compound, whose formula is shown below, is reddish-violet to reddish-brown in colour.



Both these methods were used by Gedigk and Pioch (1956) in their studies on metal-binding by connective tissues. They found that localization of silver (AgO) was better with the rubeanic acid method since silver rhodanate was sufficiently soluble to diffuse away from its original site.

Methods for Tin

Occasionally dust from ores containing tin is inhaled into the lungs where it may produce a type of pneumokoniosis. The chief form in which tin is mined is cassiterite (SnO_2), the other forms being mixed ones containing other metals such as stannite (Cu_3FeSnS_4) and cylindrite ($Pb_3FeSn_4Sb_2S_{14}$). The problem therefore usually resolves itself into demonstration of stannic oxide.

There are three possible methods, all mentioned by Feigl (1954) in his volume on the inorganic applications of spot tests. The first of these, *the morin method*, has already been mentioned (p. 690) as a method for calcium and aluminium. The second (Eegriwe, 1940) is based on the fact that in the presence of strong mineral acids stannic salts form orange lakes with 1,2,7-*trihydroxyanthraquinone*, and similarly, in the third method (Kuznetsov, 1941) a blue-violet lake is formed with the red dye *anthraquinone*-1-*azo*-4-*dimethylaniline hydrochloride*. I have tested these three methods on human lungs containing inhaled

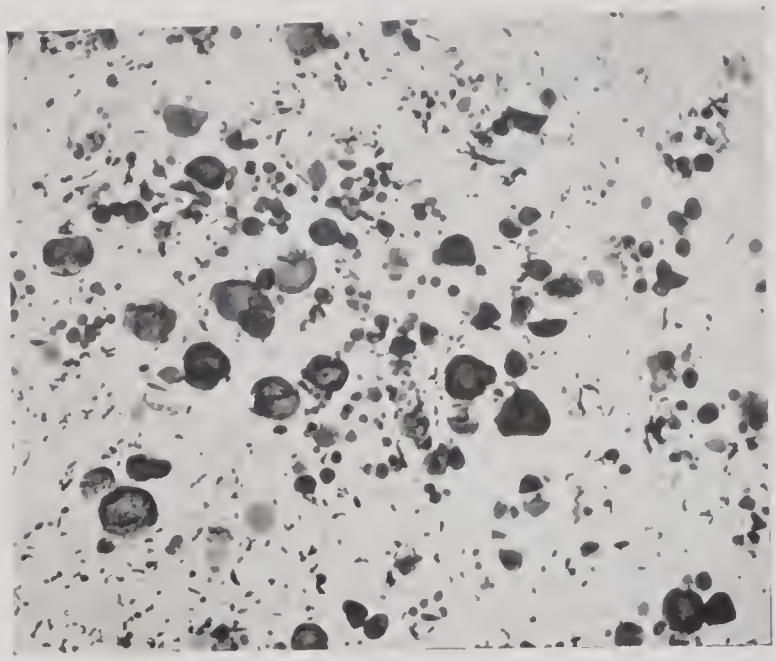


FIG. 225. *Limnaea stagnalis* (water snail). Deposits in the connective tissues containing large amounts of magnesium salts, stained bright red. Titan red method. × 120.

FIG. 226. As Fig. 225. Magnesium deposits stained bright blue. Magneson method. × 120.

[*To face p.* 704.

FIG. 227. Rat pancreas. Single islet of Langerhans. Zinc salts appear red. Dithizone method. × 130.

FIG. 228. Rat prostate. Dorsal lobe. Shows high content of zinc in glandular epithelium and in secretions in the lumen. Dithizone method. × 188.

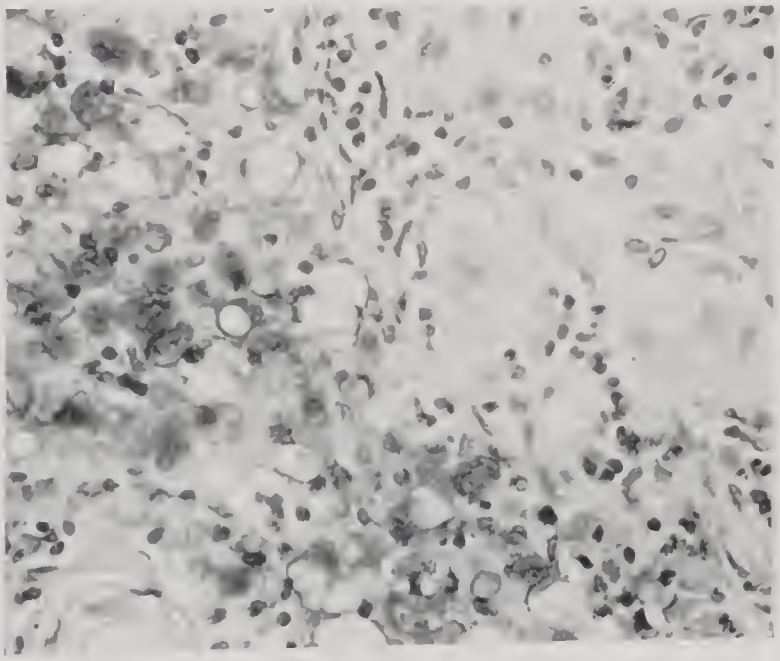


FIG. 229. Human lymph node. Gland draining area of subcutaneous tissue into which polyvinylpyrrolidone had been injected. Shows, *right*, giant cells free from deposits and, *left*, macrophages containing pink and red masses of PVP. Chlorazol fast pink. × 385.

tin ores and on rat lungs with very large amounts of oxides of tin in the form of discrete deposits. In no case could a positive reaction be obtained.

Other tests for tin which can be employed after reduction of stannic to stannous salts are the *cacotheline method* (Gutzeit, 1929) and the *diazine green S method* (Eegriwe, 1928). With neither of these have I been able to demonstrate the presence of powdered tin-containing ores artificially insufflated into the lungs of rats.

After introducing soluble tin salts into rats in various amounts Khau Van Kien and Thaï-Tuong (1956) made experiments with three staining reactions in order to determine the optimum type of fixation for tin in rat tissues. They found formaldehyde vapour to be the best, closely followed by Carnoy's fluid and alcohol. Their three methods were : (1) Dithiol (toluene-3,4-dithiol), used as a 2 per cent. solution in water or dilute HCl ; (2) Acetone-H_2S used specifically at pH 2·0 ; and (3) Mayer's hæmatein [sic] or an old solution of hæmatoxylin. These authors claimed that all forms of tin could be demonstrated and that the hæmatein reaction was the most sensitive in practice. The dithiol reaction, on the other hand, was the most specific, but it gave a red precipitate only with tin in mineral form. I have no experience of the three methods of Khau van Kien and Thaï-Tuong, but it would appear that these demonstrate only the complexes formed by soluble tin salts with tissue proteins.

Methods for Zinc

Zinc is present in the tissues as an essential component of insulin and of the enzyme carbonic anhydrase. A purified sample of this enzyme obtained from the ox contained 0·3 per cent. of zinc, equivalent to 0·2 μg. per unit of enzyme (Fischer, Tikkala and Mawson, 1955). These authors, following up earlier work by Mawson and Fischer (1952a and b), have also shown that the amount of zinc in the dorsolateral prostate of the rat increases rapidly during infancy to reach a level of 250–300 μg. per gram at 160 days. This is ten times the amount

TABLE 53

Enzyme and Zinc Levels in Rat Prostate. (*After Fischer* et al.)

Moiety	Ventral	Dorsolateral	Dorsal	Lateral
Zn (μg./g.)	13·7	180	103	590
C.A. (units/mg.)	0·11	10·5	5·1	16·0
Ac. Ph. (units/g.)	14·8	11·1	9·6	12·4
Alk. Ph. (units/g.)	53·9	21·0	16·5	23·6

present in other tissues. Carbonic anhydrase activity increased in the same way up to a maximum at about 200 days. In spite of a constant relationship between the two the very large amount of zinc in the prostate could not be correlated directly with the level of carbonic anhydrase. Nor were the levels of acid or alkaline phosphatase found to have any relationship to zinc levels, as the table on p. 705 shows.

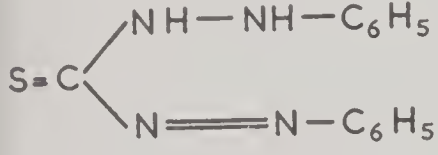
Assuming that rat carbonic anhydrase contains the same amount of zinc as in the ox it can be calculated that at 0·2 μg./enzyme unit the amount of zinc directly related to carbonic anhydrase activity in the dorsolateral prostate is 6·3 μg. per gram or 3·5 per cent. of the total. The function of the remainder is unexplained.

Using human prostate glands Hoare, Delory and Penner (1956) determined the levels of zinc and of acid phosphatase on different physiological and pathological states. They found that the normal gland contained 601 μg./gram of tissue, while in benign hyperplasia the figure was 460 μg./gram (mean values). Although the amount of acid phosphatase could be directly related to the amount of glandular tissue present there was no such relationship in the case of zinc. In human semen Mawson and Fischer (1953) found a high concentration of zinc, and they recorded the fact that spermatozoa contain more zinc than any other human tissue.

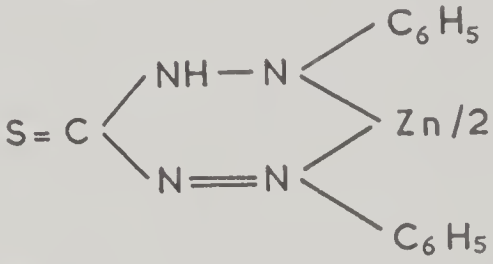
Some other associations of zinc are worthy of note. In the melanin-pigmented ocular tissues of some fresh-water fishes Leiner and Leiner (1944) found very large amounts of the metal, and Quinlan-Watson (1953) records a fall in aldolase level in oats and clover plants suffering from zinc deficiency. Hoch and Vallee (1952) described two zinc-protein complexes in the leucocytes and, in a later paper, Vallee, Hoch and Hughes (1954) recorded the properties of a soluble zinc-containing protein from human leucocytes. This protein had no carbonic anhydrase activity nor (see below) did it act as an alcohol dehydrogenase or as a carboxypeptidase. Vallee and his associates were among the first to record the existence of zinc-protein enzymes other than carbonic anhydrase, and Tupper, Watts and Wormall (1954), in a paper on the uptake of ^{65}Zn by avian eggs, recorded that zinc might be a specific activator for several enzymes. Hanson and Smith (1949) described the activation of carnosinase by zinc and similar activation has been reported for peptidases of various kinds (Johnson, 1941; Yudkin and Fruton, 1947; Smith, 1948). The carboxypeptidase of ox pancreas has been shown by Vallee and Neurath (1955) to be a zinc metalloprotein, containing 1,870 μg. per gram of zinc, and Vallee and Hoch (1955) have found a similar amount of the metal in crystalline yeast alcohol dehydrogenase. Zymohexase (Warburg, 1949) and some types of phosphatase (Cloetens, 1942) have also been found to be zinc-activated enzymes. It is evident that biological interest in zinc, and

particularly in its association with various enzymes, is at present considerable.

Zinc can be demonstrated histochemically in the islets of Langerhans (Fig. 227, p. 705) and in the prostate (Fig. 228, p. 705) of many species by the original method of Okamoto (1942a and b, 1943) using diphenylthiocarbazone (dithizone). This forms a deep red inner complex salt with zinc ions, especially in alkaline solutions :



Dithizone



The original method was used as an intravital reaction by Wolff and Ringler (1954), who were able to demonstrate zinc in the islets of the rabbit, dog, rat, mouse and guinea-pig in this way. In order to increase the specificity of the method for zinc Okamoto, Aoyama, Ibaraki, Narumi, Shibata, Kawasaki, Shibata and Komatsu (1951) used ammonium chloride (pH 8·4–8·9) and ammonium carbonate. They claimed that the former prevented both zinc and cadmium from reacting with dithizone, and the latter zinc only. Mager, McNary and Lionetti (1953) introduced for the same purpose the use of Vallee and Gibson's (1948) complex-forming buffer, which prevents the formation of metal-dithizone complexes other than zinc. Control sections were treated with dithizone in this buffer and compared with those stained with dithizone in 60 per cent. acetone.

If cold-knife or cold-microtome sections are used, and these are undoubtedly best for the demonstration of natural tissue zinc, acetone or alcohol in high concentrations in the medium causes considerable shrinkage artifact when free-floating sections are used. For freeze-dried sections and mounted cold-microtome sections, on the other hand, the acetone-dithizone reagent of Mager *et al.* is excellent. Alternatives are therefore given in Appendix 24, p. 946, one or other of which should prove suitable for most materials.

The fact that fat in sections is stained (pink) by the dithizone method was noted by Mager and his associates, and in order to distinguish this type of non-specific staining they treated sections with a solution of versene (ethylenediamine tetra-acetic acid) before applying dithizone. Since versene is a non-specific metal chelator it was assumed that a positive result reversible by this reagent was due to metallic causes.

By the use of one or other modification of the Okamoto method zinc present in the tissues at a concentration of 50 μg./gram or higher

can usually be demonstrated histochemically. Lower concentrations will be positive if the metal is localized in small intracellular components. An outstanding anomaly, however, is the behaviour of the leucocytes which are known to contain a large amount of zinc. Vallee *et al.* (1954) found 3,000 μg./gram in their soluble protein from human leucocytes. Although Chauncey and Lionetti (1952) reported positive results in leucocytes with the dithizone technique these were later found to be reproducible only irregularly.

An older method for demonstrating zinc in the tissues, used by Mendel and Bradley (1905) in their studies on the hepatopancreas in *Mollusca*, depended on the treatment of paraffin sections with *sodium nitroprusside*, and afterwards with sodium or potassium sulphide. An intense purple colour indicated the presence of zinc and the reaction was regarded by Lison (1953) as sufficiently specific. The nitroprusside method was used by Sebruyns (1946) for localization of the metal in mice which had received various doses of ZnO by mouth. As an alternative an *alizarin lake method* attributed to Crétin (1933) was employed.

In view of the excellence of the dithizone method the nitroprusside method finds little use in modern histochemistry and neither it nor the alizarin lake method can be recommended. A number of oxidation catalytic reactions for zinc salts have been described by Feigl (1954), the most sensitive (0·1γ Zn) makes use of the fact that zinc ions raise the oxidation potential of ferricyanide by removing the ferrocyanide formed during the oxidation of a variety of compounds. For this purpose 3,3′-dimethyl-naphthidine has been recommended. When this method is applied to sections of pancreas and prostate, and to invertebrate tissues containing large amounts of zinc, no oxidation occurs.

Methods for Potassium

The method of Macallum (1905) for the demonstration of potassium was based on the formation of an insoluble triple salt by the application of sodium cobaltinitrite to potassium salts in fresh tissue sections. The orange-yellow crystalline precipitate of sodium potassium cobaltinitrite was then converted into cobalt sulphide with yellow $(NH_4)_2S$. A large number of modifications of Macallum's method have been devised. Menten (1922) observed that the maximum precipitate was obtained after 2 hours, but preferred to stain for 15 minutes only. Rohdenburg and Geiger (1928) placed small tissue blocks in the cobaltinitrite solution for 1 hour, afterwards washing for 20 minutes and then placing the tissue in ammonium sulphide. Poppen, Green and Wrenn (1953) found that this last method was unacceptable because it was impossible to remove the large excess of precipitate in

the interstitial tissues by washing. They employed sections from heart muscle fixed in alcoholic formalin for 48 hours. Sections were cut at 7 μ and dewaxed in xylene followed by two changes of absolute alcohol. They were then transferred directly into the solution of cobaltinitrite.

The freeze-drying method of preparing tissue sections overcomes the problem of diffusion of potassium ions up to the stage at which an aqueous solution can be applied to the section. Gersh (1938) therefore evolved a modified cobaltinitrite method, for application to 15 μ freeze-dried sections, in which elaborate precautions against this diffusion were taken. Observing that potassium cobaltinitrite is soluble in water at room temperatures, but scarcely at all at 0°, he employed this temperature for the reaction. Crout and Jennings (1957) compared the original method of Macallum with that of Poppen *et al.* and with the method of Gersh. They observed that sections stained by the latter method showed a fine dense granular precipitate, evenly distributed throughout muscle cells, whereas with the other two methods the precipitate was irregularly distributed and the granules much more coarse. By means of potassium analyses carried out in parallel with the histochemical studies it was shown that all the potassium present in the tissues is precipitated by cobaltinitrite. Crout and Jennings varied Gersh's method in respect of some minor details, and these, as well as the original method, are given in Appendix 24, p. 944.

The reaction of Carere-Comes (1938), using dipicrylamine, is quite useless. This dye (aurantia) stains basic proteins independently of their potassium content.

Methods for Sodium

The zinc uranyl acetate method (Kolthoff, 1927), which is widely used for spot tests (see Feigl, 1954), cannot be applied to fresh frozen sections with any hope of successfully localizing the sodium ions. When it is applied to such sections or to freeze-dried material and examined under the fluorescence microscope the tissues exhibit a general fluorescence, and it is impossible to say how much of this is related to sodium and how much is non-specific. No other methods for sodium have been applied histochemically.

Histochemistry of Inorganic Anions

Methods for Phosphate and Carbonate

Provided that phosphate and carbonate are present as insoluble calcium salts they can be distinguished very simply by mounting the section containing them in water and running glacial acetic acid under one edge of the coverslip while the preparation is examined under the microscope. The deposit, easily seen with the condenser slightly out

of focus, dissolves quietly if it is phosphate and with evolution of bubbles of CO_2 if carbonate. A mixture of the two salts is not distinguishable, but CO_2 can be positively identified by the method of Bunting (1951) which is given in Appendix 24.

Molybdate methods can be used to demonstrate both organic and inorganic phosphates, but in the first case the conversion of the organic salts to inorganic phosphate is a necessary preliminary to their demonstration. As mentioned in Chapter I, the molybdate methods are variations of the ammonium molybdate technique of Lilienfeld and Monti (1892). A useful modification is that of Serra and Queiroz Lopes (1945) (referred to in Chapter VIII, p. 190), which, like the other methods, gives a blue colour with phosphate. Although the accuracy of localization of organic phosphate by this method is very low, preformed inorganic phosphate deposits are better demonstrated. Fig. 211 shows the application of a modified Serra technique to a lymph node containing giant cells with doubly refractile crystals of calcium phosphate (Fig. 210). In this case the crystals only stained at their free borders, but amorphous phosphate in the surrounding cytoplasm showed brilliantly. It is probable that much of the latter was due to diffusion of phosphate ions during performance of the test.

Cheng (1956) employed a modification of the method of Lowry and Lopez (1946) for demonstrating inorganic phosphate in the presence of labile phosphate esters. In his technique (see Appendix 24, p. 945) short formalin fixation was employed, and frozen sections, but loss of inorganic phosphate into the fixative was noted to be a serious factor.

Methods for Chlorides

A large number of authors have reported work on the histochemical localization of chloride, using silver techniques exclusively. Lison (1936b) sought to avoid the gross errors of earlier methods using tissue blocks by the intravascular injection of the silver solution. He showed that silver salts could penetrate only a few cells deep in a block of tissue after 4 hours' incubation and, since chloride ions are extremely diffusible, localization was obviously impossible.

Lison (1953) is extremely critical of the silver reaction for chloride in histochemistry. He points out that the reaction is an indirect one, since the final product (metallic silver) is related to the total number of silver ions in the tissue and not to the number of chloride ions. If traces of silver remain in the tissues false localization will occur. These can best be avoided by washing sections (not blocks) with dilute nitric acid in place of distilled water. Chloride bromide and iodide of silver are insoluble in dilute mineral acids, phosphate and other salts are removed. According to Lison nitric acid has two other advantages.

First, it dissolves out metallic silver precipitated in the tissues by the direct action of reducing agents, such as ascorbic acid, and secondly, it releases silver which has been adsorbed on the tissue proteins and lipoproteins.

Gersh (1938) evolved a procedure for localizing phosphate (phosphate-carbonate) and chloride in freeze-dried sections. He employed two solutions of 60 per cent silver nitrate, one containing phosphoric acid and saturated with silver chloride, the other containing non-phosphoric acid and saturated with silver phosphate and chloride. The first precipitates chloride only, the second both phosphate and chloride. The sections are examined after short treatment with ultra-violet light.

This method is free from most of the objections which can be applied to the tissue block methods, although it must be remembered that chloride ions can and probably do diffuse during the process of freeze-drying (see Chapter III, p. 32). As pointed out by Lison, however, Gersh's method in its original form lacks specificity since no precautions are taken to remove silver ions from the sections before blackening with ultra-violet light. According to Gersh there is a period during which only silver phosphate and chloride are reduced to metallic silver, before the general reduction of silver nitrate by tissue proteins occurs. This conception, based on the progressive darkening of the sections, is inadmissible and I agree with Lison that the method should be modified before the localization of chloride which it affords can be accepted as accurate.

Methods for Iodides

Inorganic iodides have not been localized successfully by histochemical methods other than those employing an autoradiographic technique (see Chapter XXVII, p. 742). Gersh and Stieglitz (1933) and Lison (1936a and 1953) concluded that it was impossible to localize diffusible iodide by means of precipitant reactions. These have also been largely unsuccessful.

One method, that of Turchini (1930), may be mentioned. This author demonstrated bound iodides in the tissues of the oyster by treating them with nitrous acid (10 per cent. sodium nitrite in 1 per cent. sulphuric acid). This released iodine in elemental form which stained the tissues yellow. Treatment with starch solution produced a blue colour. According to Lison (1953) only weakly bound iodine can be demonstrated by this method.

McAllister (1953) has described a method which gives a red fluorescence in the presence of iodides. This depends on their reaction with 1-methyl-2-mercaptoimidazole and bismuth acid sulphate

($BiH(SO_4)_3$). The possibility of adapting this reaction for histochemical use might be considered.

Conclusion

Because of the fact that many of the metals dealt with in this chapter can be demonstrated by a variety of methods I have set down, below, a list of metals for which more than a single workable method exists. The order in which the methods are given is to some extent the order of choice, for mammalian and human pathological tissues, but it is obvious that no hard and fast rules can be laid down concerning the choice of method except in rare cases.

Aluminium

Neutral solochrome azurine (p. 935).
Acid solochrome cyanine (p. 936).
Naphthochrome green (p. 937).
Morin-fluorescence (p. 935).
Aluminon (p. 935).
Solochrome-fluorescence (p. 936).

Beryllium

Alkaline solochrome azurine (p. 938).
Alkaline quinalizarin (p. 938).
Naphthochrome green (p. 937).
Neutral solochrome azurine (p. 935).
Autofluorescence.
Ehrlich's hæmatoxylin.

Calcium

Morin-fluorescence (p. 935).
Phthalocyanin (p. 933).
Gypsum method (p. 933).
Alizarin red (p. 932).
Calcium red (p. 933).
Alkaline quinalizarin (p. 938).
Naphthochrome green (p. 937).

Copper

Rubeanic acid (p. 940).
Dithyldithiocarbamate (p. 940).
Oxidation reactions (p. 940, 941).
Dithizone (p. 946).

Lead

Rhodizonate (p. 942).
Sulphide (p. 941).
Chromate (p. 942).

Zinc

Dithizone (p. 946).
Nitroprusside.

Nickel

Rubeanic acid (p. 940).
Dimethylglyoxime acid (p. 939).

Iron

Prussian blue (p. 931).
Turnbull blue.
Dinitrosoresorcinol (p. 931).

Silver

Dimethylaminobenzylidene rhodanine (p. 943).
Dithizone.

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CHAPTER XXV

EXOGENOUS AND MISCELLANEOUS ENDOGENOUS SUBSTANCES

This chapter concerns the localization of a number of substances and materials which have little in common, except that they are often introduced into the human and animal body for therapeutic or other reasons. Subsequently, they may appear in tissue sections and require identification by histologists and histochemists.

The first two substances, dextrans and polyvinyl pyrrolidone (PVP), are used as plasma substitutes but, unfortunately, they escape rapidly from the blood stream and find their way into the tissues. Polyvinyl pyrrolidone has also been employed as a vehicle for various pharmaeologically active materials which must be given by subcutaneous or intramuscular injection. Dextran sulphate is an anticoagulant which has been used as a substitute for heparin.

A number of textile fibres may find their way into the tissues when used as surgical ligatures and the commonest of these are linen or cotton thread, silk or nylon. Other materials for which operative interference is responsible include gelfoam (fibrin foam) alginate, polyvinyl alcohol and of course talc, which produces a characteristic granuloma.

The trypanocidal drugs, Suramin and Germanin, are two examples of pharmaceutical products which can be localized in the tissues by histochemical methods after administration in the therapeutic doses. The list of substances in this category might well be made larger by including all those drugs which are fluorescent and which can be localized by the simple expedient of examining sections containing them under the UV microscope. These include various acridine and quinoline derivatives, such as quinacrine (atabrine, mepacrine), some of the sulphonamides and a number of pteridines. Substances introduced for experimental purposes include all kinds of metallic dusts, powdered ores and other industrial products. Some of these have been considered in Chapter XXIV, and others do not warrant consideration in this chapter.

Dextran and Dextran Sulphate

The deposition of high MW dextrans in rabbit kidneys was first described by Goldenberg, Crane and Popper (1947), but many authors subsequently failed to find any change in the kidney or other organs and no accumulation of dextran in the cells of the RE system (Ingleman,

1947, 1949; Thorsen, 1949; Bull *et al.*, 1949). Partially hydrolysed dextrans, whose molecular weight varied from 28,000 to 400,000, were injected into rabbits by Friberg, Graf and Aberg (1951). By means of the PAS reaction these authors were able to demonstrate dextran in the glomeruli and in the cells of the convoluted tubules and loops of Henle. Excreted dextran was found in masses in the lumen of the collecting tubules. Further histochemical studies were made by Persson (1952a and b), who also used rabbits. He fixed his materials in a variety of fixatives, of which the best was found to be a mixture of equal parts of ethanol and methanol. This precipated the dextrans very rapidly. A 3 per cent. solution of sulphosalycylic acid in methanol was also found to be effective. Persson tested a number of oxidation procedures and followed these in each case by treatment with Schiff's solution. He found that periodic acid was the most satisfactory. Any substance in the tissues which was strongly PAS-positive, unstained by the Bauer-Feulgen procedure, blue with Azure 1 after HIO_4 and only light yellow with iodine, was regarded as dextran. Apart from the kidneys, dextran was found in a number of other sites in the body, particularly in the cells of the R.E. system.

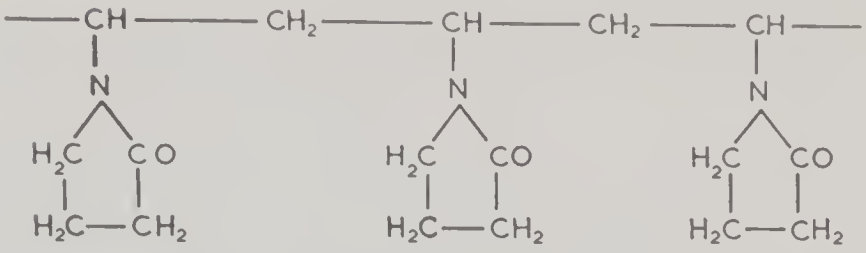
Mowry, Longley and Millican (1952) were the first to take adequate precautions against loss of the water-soluble dextrans during the PAS routine. These authors fixed thin blocks of tissue in absolute alcohol and avoided contact with water thereafter by floating out their (paraffin) sections on warm 95 per cent. alcohol and by using alcoholic periodic acid and alcoholic Schiff's reagent. Details of their method are given in Appendix 25, p. 947. Further work with this method was reported by Mowry and Millican (1953).

A method for the demonstration of dextran in the tissues evolved by Jancsó-Gabor was described by Jancsó (1955). Methyl alcohol-fixed fresh preparations of tissues were stained with Niagara sky blue in 90 per cent. methanol for 15–20 minutes. A different problem is the demonstration of dextran sulphate, also mentioned by Jancsó. For this purpose Mowry used a descending series of alcohols containing toluidine blue. This dye forms a precipitate with dextran sulphate which is insoluble in water, and it is thus possible to proceed from the alcoholic toluidine blue solution to the aqueous solution in which the maximum degree of staining can be achieved. Details of this method are given in the Appendix (p. 947). A recent study (Vickery, 1956) of the fate of dextran in the human subject is based entirely on the use of Mowry's techniques.

Polyvinyl Pyrrolidone (PVP)

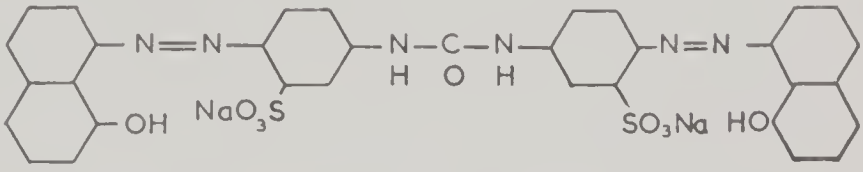
The storage of this substance, whose formula is given below, was described in human tissues by Nelson and Lusky (1951), Husselmann

(1952), and also by Jeekeln (1952). Later studies were made by Janesó *et al.* (1953), Janesó (1954) and by Freiman and Gall (1955). A number of staining methods give satisfactory results. The simplest



is the iodine method (Janesó, 1954), but this lacks permanence. Congo red stains PVP a deep eherry-red eolour and Freiman and Gall reeommend a technique based on this stain. Unfortunately Congo red staining of PVP is also impermanent, since fading takes place within a few days.

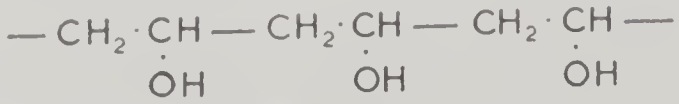
The best and most permanent method in my hands has been Janesó's Chlorazol fast pink method. The formula of this dye is somewhat similar to that of Congo red (see p. 285) and it stains PVP a pale pinkish-red eolour.



All the above methods are given in Appendix 25, p. 948. The results of the last method are illustrated in Fig. 229, p. 705, where the deposits of PVP in a regional lymph node adjaeent to a focus of PVP injection are elearly shown. A report on this material has been made by Towers (1957), and Frommer (1956) has described the effect of intravenous PVP on the eells of mouse liver.

Polyvinyl Alcohol

This polymer is less often found in human tissues than PVP, but tubes composed of a sponge-work of the eompound have been used in arterial grafting. The graft stains blaek with Weigert's elastic stain, which should surprise nobody if its formula (below) is considered in the light of modern views (Chapter VII, p. 165) on the staining of elastic tissue. Polyvinyl alcohol also stains black with iodine and red with Chlorazol fast pink.



Polyvinyl alcohol.

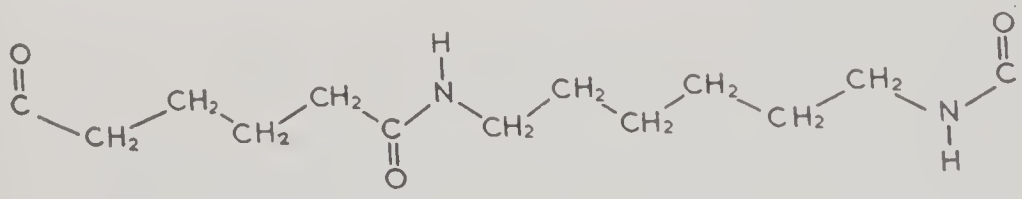
Suramin

A method for demonstrating this substance in the tissues was reported by Jancsó and Jancsó-Gabor (1953), which depended on simultaneous fixation of freshly teased tissues with methanol containing methylene blue eosinate (May-Grunwald stain). Other dyes capable of forming a precipitate with Suramin were phenosafranin, cresyl blue, and Azure B. With any of these dyes it was essential to avoid contact with water and, after a few minutes in the methanolic stain, differentiation was carried out by agitation in absolute alcohol followed by clearing in xylene and mounting in Canada balsam.

Textile Fibres

Due to the presence of side groups which are highly polarizable, cellulose nitrate and polyacrylonitrile (Orlon) show negative birefringence in polarized light. Because of its crystalline nature, nylon suture material in tissue sections is also birefringent, as are fibres of lint or cotton thread. Polarization microscopy is therefore of little use from the diagnostic point of view. Venkataraman (1952) gives a differential table for the identification of textile fibres and some of the methods suggested therein may be found useful histochemically. Silk and nylon, he says, are soluble in conc. HCl while cotton is not. Silk and cotton, on the other hand, are soluble in ammoniacal copper solutions while nylon is not. Strong solutions (90 per cent.) of phenol dissolve nylon alone. A large number of stains for the primary wall of the cotton fibre are given by Moore (1953). These include ruthenium red, since the primary wall contains pectic acid (see Chapter IX, p. 263) as well as cellulose.

Special staining techniques exist for polyamide fibres like nylon which contain a number of free carboxyl groups, together with a smaller number of amino groups and some terminal amides. The formula of a linear polyamide molecule given below suggests that the free carboxyl groups would be likely to play an important part in staining reactions.



It has been found, however (Vickerstaff, 1954), that the terminal amide groups are of great importance, especially in the staining of nylon by acid dyes.

Differential stains for the histochemical identification of nylon have not been investigated. Dyes of the type known as " dispersed,"

that is to say, water-insoluble anthraquinone dyes employed as aqueous suspensions, can be used to stain nylon fibres. Since the drawn nylon fibre is highly crystalline the dispersed dyes, combined with the amide groups, are orientated along the fibre. This produces dichroism when the stained fibre is viewed by polarized light. Many soluble dyes have been used to stain nylon fibres, but these stain cotton and silk even more readily and are thus useless for differentiation. Although the need for histochemical distinction between the various natural and synthetic fibres is not very great, a definitive study of the staining and other properties of all materials likely to be found in tissue sections would be of considerable value to histopathologists.

Miscellaneous Endogenous Substances

Urea

The only specific test for urea depends on the formation of crystals of dixanthydrol urea by fixing fresh pieces of tissue in xanthydrol in alcoholic acetic acid. Rosettes of yellowish-brown needle-shaped crystals indicate the localization of urea. According to Gomori (1952), the usefulness of this test in histochemistry is limited because of damage to the structure of the tissues by the strong acetic acid and by the amount of diffusion which occurs during the reaction. Details are nevertheless given in Appendix 25, p. 949. Lillie (1954) modified the method by performing the first stage of the reaction for 14 days at −25°. He considered that further study was necessary before the method could be used routinely.

Feigl (1954) suggests the production of a sensitive test for urea by condensation with phenylhydrazine to form diphenylcarbazide. In the presence of certain metals (e.g. nickel) an insoluble highly coloured complex would be produced. Unfortunately it seems that the conditions for urea-phenylhydrazine condensation are unlikely to be realized histochemically.

Uric Acid and Urates

In the tissues uric acid is normally present in the form of crystalline acid sodium urate. The crystals are slightly soluble in dilute alkali, but insoluble in all ordinary reagents. Urate deposits can be distinguished from phosphate and carbonates by their solubility in dilute solutions of lithium carbonate. They possess two histochemical properties, basophilia and argentaffinity, and tests for their demonstration depend on one or the other of these. Of the two the silver methods are the most popular. Lillie (1954) gives both De Galantha's (1935) silver nitrate method and Gomori's hexamine-silver technique and this last method is reproduced in Appendix 25.

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CHAPTER XXVI

FLUORESCENCE MICROSCOPY

Introduction

Although the ultra-violet microscope has been used for histological and histochemical purposes for many years, there has recently been a considerable revival in interest in fluorescence microscopy and in its employment for a number of new techniques. This is partly due to the impetus given by the fluorescent antibody methods (Chapter VI) whose general adoption has meant that high resolution ultra-violet mieroseopes are now to be found in many institutes which formerly possessed none. Partly, however, it is due to the increasing realization that, despite its many disadvantages, fluorescenee microscopy is capable of raising the sensitivity of any histochemical method whose final product in the tissues is fluorescent, or can be modified so as to be fluorescent. Rapid development along these lines can confidently be expected.

I have no intention here of writing either a comprehensive review or a treatise upon the optics of fluoreseence microscopy. Many excellent reviews have been written on the subject and the reader is referred to these for fuller information (Hamperl, 1934, 1935, 1947 ; Popper, 1944 ; Glick, 1949 ; Popper and Szanto, 1950 ; Richards, 1955 ; Price and Schwartz, 1956). It is my intention briefly to detail the current uses of fluorescence microscopy, in order to put the subject into perspeetive in applied histochemistry, and to add some details which eannot be found elsewhere.

The earlier history and applieation of fluorescenee microscopy are to be found in the works of Dhéré (1933), Haitinger (1938), Ellinger (1940), Strugger (1949) and Braütigam and Grabner (1949). These, and the references given above, should be sufficient introduction to the subject for the majority of applied histochemists.

Advantages and Potential Uses of Fluorescence Microscopy

The main advantage of the method, which transcends all others, is its great sensitivity. This is coupled with the effect of high contrast which it gives. As Price and Schwartz (*loc. cit.*) have pointed out, a 1 per cent. difference in transmitted light between two different areas of a cell can be demonstrated by fluoreseenee as a 100-fold difference. The second advantage, which follows from the first, is that very low concentrations of the excitable material are necessary to provide

visible fluorescence and fluorescent compounds can thus be added to living cells in quantities too small to produce toxic damage.

There are a number of disadvantages inherent in the method, in addition to those connected with the material difficulties of providing the proper apparatus. Because fluorescent light is emitted in all directions from its point of origin in the tissues the degree of resolution obtainable is less than that given by visible light methods. Secondly, quantitation is not easy. This is partly due to the fact that fluorescence is very susceptible to interference by physical and chemical factors. Quantitation has nevertheless been attempted by Mellors *et al.* (1952) using densitometric methods. It seems that measurements of the emitted light to within 10–30 per cent. should be possible. Greater accuracy than this, which is nevertheless within the range most useful to applied histochemists, is not at present to be achieved.

The potential uses of fluorescence microscopy fall into two main divisions. These are (1) *Primary fluorescence* and (2) *Secondary fluorescence.* The first concerns the observation of naturally occurring fluorescent structural materials in plant and animal tissues and the localization of any fluorescent products of their metabolism. Secondary fluorescence studies are divisible into five groups :

(1) *Fluorochroming or Fluorescent Staining.*
(2) *Fluorescent End-product Methods.*
(3) *Fluorescent Tracer Methods.*
(4) *Foreign Substance Detection.*
(5) *Fluorescent Antibody Methods.*

This last group of methods has been fully considered in Chapter VI and will not further be considered here. Before presenting some details of recent applications in the other fields, however, it may be expedient to discuss some of the factors connected with the provision of suitable conditions for the various types of fluorescence microscopy.

Physical Conditions

The provision of suitable apparatus is considered fully in the papers of van Duijn (1953–54, 1954–55), Gottschewski (1954), Richards (1955), and Price and Schwartz (1956). It is easy to get advice on the subject from the manufacturers of fluorescent microscopes but it is not always easy to distinguish the relative merits of the available apparatus and, since many are apparently designed with a single purpose in mind, the general histochemist may well feel that it is better to construct for himself an apparatus capable of modification for use with a variety of different techniques.

I propose to consider the physical characteristics of fluorescence microscopy under the following headings. (1) *The Nature of Fluorescence*, (2) *The Microscope*, (3) *Illumination*, (4) *Filters*, (5) *Optimal Image Production*, (6) *Photography*, (7) *The Preparation of Sections.*

The Nature of Fluorescence

Those who require detailed observations on this subject are referred to the paper by Rosenberg (1955) in which the principles and kinetics of photochemistry and luminescence are dealt with in a full and competent manner.

Certain substances which absorb incident radiation of various wavelengths convert some of the absorbed energy into visible light of longer wavelengths. If this emission occurs with negligible delay the phenomenon is known as fluorescence. If, on the other hand, it continues after the exciting radiation has been interrupted, it is known as phosphorescence. Since all the absorbed energy need not be converted into visible light the relationship between the quanta absorbed and the quanta emitted is called the " fluorescence yield," or " fluorescence efficiency " of the substance concerned. This is an important factor, especially in designing and synthesizing suitable fluorescent couplers for labelling proteins and other substances. It is of less importance in relation to the techniques of fluorochroming, however, since here it is possible to attach to tissue components a relatively vast excess of the fluorescent substance.

The spectrum of the absorbed light, in relation to a fluorescent substance, usually shows distinct bands which represent those wavelengths which are particularly efficient in excitation of the fluorescent response. This factor is also of great importance, though often overlooked by the possessors of apparatus designed to deliver light of fixed wavelength. The spectrum of the emitted light may show a peak, which accounts for the characteristic colour of the fluorescence, but in other cases it is not sharply defined. This is the condition observed with most of the bluish-white fluorescent tissue components.

In low concentrations, the intensity of the fluorescence is proportional to the amount of material present and, as mentioned above, the method is thus a highly sensitive one for the detection of minute amounts of material. Unfortunately, fluorescence is easily modified by relatively minor changes in pH, and by the presence of small amounts of impurities (e.g. Hg^{2+} or Os^{4+}). These are known as " quenchers ".

Conditions for Observations of Fluorescence. In certain instances fluorescence is so great that it can be recognized in diffuse daylight, as with certain fluorescent dyes used in advertisements and lipsticks.

This degree of fluorescence is seldom encountered in fluorescence microscopy. Microscopically there are two main levels at which fluorescent phenomena may be observed, those involving moderate fluorescence (i.e. secondary fluorescence after the use of fluorochromes) and those involving much fainter fluorescence. The latter require meticulous attention to detail in the provision of suitable apparatus and in the preparation of the specimen. In this category are certain types of autofluorescence, secondary fluorescence from small objects like acid fast bacteria (stained with Auramine O), and fluorochromes present in extremely low concentrations as in the fluorescent antibody techniques.

The amount of light involved in fluorescent studies is always relatively low by comparison with that falling on the object so that especially in visual work, the aim must be to increase contrast by eliminating all other light from the field of view while getting the maximum amount of fluorescent light through the objective. In photography, on the contrary, maximum intensity of the emitted light is the primary consideration and contrast is of lesser importance.

The precise equipment for any laboratory will depend on what type of work is to be undertaken and what type of apparatus can be afforded.

The Microscope. Optical glass transmits long wavelength ultraviolet light, which excites fluorescence in most tissues and fluorochromes, so that it is quite possible to use an ordinary light microscope for fluorescent microscopy. There are certain features of the standard microscope, however, which require attention or modification. First, the usual (silvered) substage mirror is a poor reflector of UVL and thus it should be replaced by a metallized front-surface mirror. Clip-on types for the usual mirrors are obtainable commercially. Secondly, the condenser should be of the simple Abbé type with as few lenses as possible. Extra lenses absorb much of the light and the balsam cement with which they are mounted may itself fluoresce and thus reduce the effective contrast of the final picture. It is usual to employ a monocular microscope since the intensity of fluorescence is seldom sufficient for observation with binocular optics. These reduce the intensity of light reaching the eye to less than half (Richards, 1955).

Illumination. It is required of the illumination system that it shall illuminate the field of view in the object and that it shall provide maximal intensity over this field. The system of " critical illumination " where the source is imaged in the field of view, is unsatisfactory for fluorescence microscopy, since the sources of UVL are too small to illuminate the whole field. Köhler's system of illumination (Fig. 230, below) is the ideal, providing precise conditions for fulfilment and precise control.

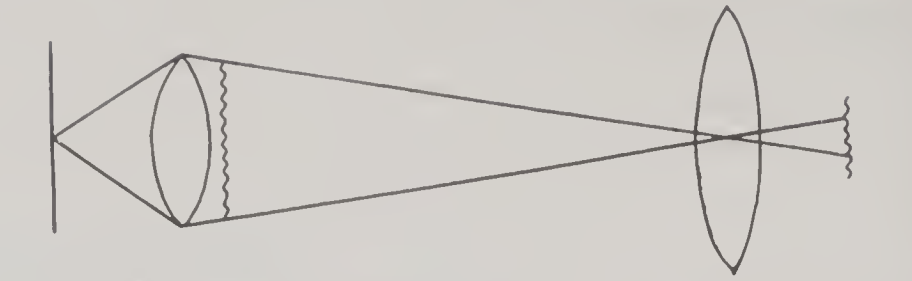


FIG. 230.

Köhler's illumination requires no more than that the source be imaged in the aperture of the substage condenser by the collector lens and that the aperture of the collector lens be imaged in the field of view by means of the substage condenser. Both substage condenser and the lamp collector should be aplanatic in an ideal system. With Köhler's illumination the diameter of the field of illumination is proportional to the aperture of the collector and the magnification of the substage condenser. The intensity of illumination is proportional to the area and brightness of the source and the square of the available NA of the condenser.

The area of illumination, as opposed to its intensity will be greatest when the collector is imaged in the field of view, as shown in Fig. 231, below.

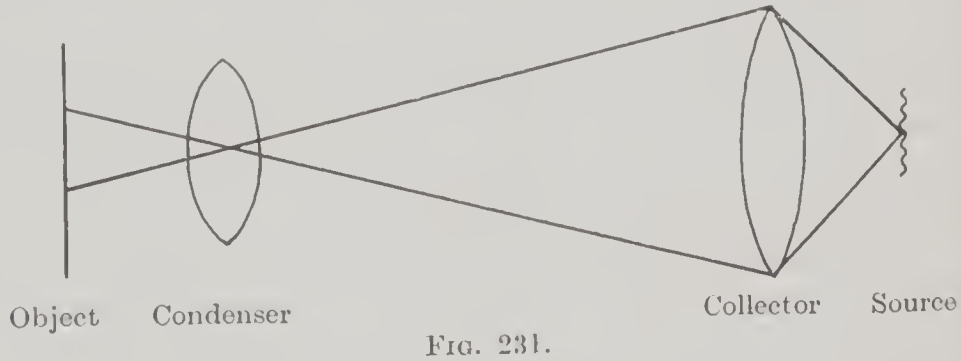


FIG. 231.

The advantages of an optical bench are apparent. This enables one to control the magnification of the collector lens and the alignment of the whole system.

Filters. For all critical studies it is necessary to employ two complementary filters. The first, situated in the illumination system, transmits the exciting wavelengths and eliminates those wavelengths which we desire to observe. The second, situated in the image-forming system, transmits the fluorescing wavelengths and absorbs the existing rays. Apart from the presence of the object emitting the fluorescent rays these two filters in combination should absorb all the visible and shorter wavelengths. In the presence of a fluorescent object, if the two filters are reversed, no fluorescence should be observed.

Any light which is observed under these conditions is to be regarded as extraneous. It is apparent that the excitation filter should give maximal transmission of the peak activating wavelengths (corresponding to the absorption spectrum of the fluorescent substance); likewise, the barrier filter should transmit all the emitted radiation while withholding all the activation radiation. Suitable UV-transmitting and UV-absorbing filters are listed in Appendix 26.

Optimal Image Production. This requires, as a primary consideration, the provision of maximal irradiation of the object with the exciting wavelength. Most incandescent sources are unsuitable for the purpose. The positive crater of the D.C. carbon arc has a brightness of about 18,000 stilb (candles/cm^2) and its brightness increases with amperage. Powerful arcs of 20 amps have been used for fluorescence microscopy but these produce a great amount of heat and light and most workers therefore avoid them, preferring arc streams as sources. These are produced by compact source mercury vapour lamps, which operate at high pressures (600 lb./sq. inch) within a shell of fused quartz. They provide a brightness of 25,000 stilbs and an arc length of 2 mm. (150 watt) or 3·75 mm. (250 watt). The source is not circular but oval. Forced cooling is unnecessary.

After providing light of maximal intensity steps must be taken to transmit it to the object with as little loss as possible. Optical glass transmits UVL down to 3500Å and, if lower wavelengths are required, it may be necessary to provide lenses etc. of special glass or quartz. The window of the lamp (if present), the heat collecting water bath (if used), the collector lens, condenser and microscope slide may all have to be constructed of such special glass or quartz. It is obviously convenient, when designing an apparatus, even if the use of wavelengths below 3500Å is not considered, to construct the above items from UV-transmitting materials.

The use of a front-surface mirror has already been mentioned but, if necessary, this item can be avoided by directly illuminating the substage condenser. The latter should be at maximum aperture since the intensity of illumination increases as the square of the numerical aperture. This may require immersion of the condenser in order to obtain apertures greater than N.A. 1·0. The condenser should be aplanatic, bringing rays of all apertures to a focus on the subject. An achromatic condenser will have disadvantages in autofluorescence which outweighs its theoretical advantages. It will be evident that to obtain maximum apertures of illumination with the substage condenser the image of the arc should fill the entrance pupil of the condenser. This requires that the collector lens shall magnify the source adequately. If it does not, there will be no advantage in having the higher available aperture in the condenser.

Fluorescent behaviour is easily modified, as mentioned above. Some substances (e.g. iodine, mercury and iron) entirely quench primary fluorescence and these should be avoided in fixatives (if used) and in any pretreatment of sections before irradiation. Other substances (e.g. Vitamins A and A_2) fade rapidly on exposure to UVL. This may be a feature of diagnostic importance but it also means that the slide must be screened, except during the period of observation, when UV-labile substances are being studied.

Fluorescence is also easily modified by changes in pH level. This is readily observed in the use of buffered fluorochromes (see below). The fluorescent colour emitted may be modified by the nature and concentration of the bound fluorochrome, whether this is cationic, anionic or neutral, and also by the nature of the binding molecule. Primary fluorescence is also modified by pH but this feature does not seem to have been used in applied histochemistry.

Maximum Numerical Aperture of Objective. The power of an objective to gather light increases as the square of the numerical aperture so that a 4 mm. objective of N.A. 0·95 will collect more than twice as much light as one of N.A. 0·65. The higher aperture objective requires a correction collar, for coverslip correction, but the dark background of fluorescence microscopy makes this very easy to adjust.

Lowest Convenient Magnification. The brightness of the image is reduced according to the square of the magnification so that the image with 5x eyepiece will be four times as bright as with a 10x eyepiece. Lower magnifications are also generally associated with an increase in the field of view. The importance of illuminating no more than the field of view cannot be too strongly stressed. A common difficulty with the lower magnifications is to illuminate the whole of this field. No single illumination system can satisfy the demands of a range of objectives between 2·5x and 100x and it is therefore most satisfactory, if at all possible, to design one's apparatus for a single objective. With Köhler's illumination (see Fig. 230) the area of the field of view depends on the area of the collector lens and the magnification of the condenser. The latter varies with the focal length of the condenser and with the distance of the collector from the substage. One can increase the focal length of the condenser by removing its top (back) lens, but this also reduces its N.A. with consequent reduction in intensity of the light passing through it. For bright ground illumination this is unimportant, since low power objectives have low numerical apertures. For fluorescence microscopy, however, maximum numerical apertures are desirable since the direction of the exciting ray is not related to the direction of the fluorescent ray. It is therefore essential, if possible, to employ a condenser of maximum numerical aperture. The diameter of the field of illumination is then regulated

by altering the position of the collector lens (see Fig. 231, p. 726). For low power objectives one requires a collector of large diameter and of very much shorter focal length than is needed for a higher power objective, if maximum illumination is to be obtained. Lesser requirements enable one to use less efficient systems and, as a rule, it will be sufficient to change the focal length of the condenser by unscrewing the front lens, putting up with the loss of the aperture previously available.

Coated Lenses. Since approximately 5 per cent. of light is lost at each interface in a system of glass lenses in air, and since such light contributes to glare, coated optics should materially benefit the performance of a fluorescence microscope. The number of surfaces should be reduced, as emphasized above with respect to the condenser. Single layer filters should be employed if possible.

Dark-Adaptation. In order to increase retinal sensitivity observations by fluorescence microscopy should be made in a properly equipped dark room from which all extraneous light can be excluded. The lamp house, which is a potent source of such light, should of course be screened.

Maximum Contrast. The human eye is sensitive to relative amounts of light, that is to say to contrast, rather than to absolute amounts. An increase in the emission of fluorescent light is of no advantage if it is accompanied by a simultaneous increase in the amount of background illumination. The requirement for maximum irradiation with the exciting wavelength, and for maximum brightness of the fluorescent image, may therefore have to be modified in order to obtain maximum contrast. Of first importance in this respect is the elimination of background light, by the use of a dark-room and by the use of truly complementary filters. That is to say, no light should pass the barrier filter. A great improvement (in contrast) is often produced by using filters of double thickness.

A second important factor is extraneous fluorescence. This can arise in many places and great care should be taken to eliminate it and to achieve a perfectly black background. Any object situated between the activation and barrier filters may be responsible and the tracking down of sources of extraneous fluorescence requires great care. The condenser may be responsible, especially if it is achromatic-aplanatic as mentioned above. Quartz condensers are usually entirely free from fluorescence, and they may be desirable on this account. The microscope slide and the coverslip may also fluoresce and, if necessary, these can be made of special UV-transmitting glass (Reichert).

The field of illumination of the section should be limited to the field of view, by means of the field diaphragm, in order to avoid lateral

scatter of fluorescence. Vertical scatter of fluorescence will occur when the thickness of the section is greater than the depth of field of the objective, and extrafocal fluorescence of this type may cause glare. Since the depth of field of an oil immersion objective is less than 0·5 μ, and of a 40x objective about 1 μ, the ideal condition of limiting section thickness to field depth is not easily obtained. Sections should nevertheless be cut as thin as possible, and they must be mounted in a non-fluorescent medium (see below). If an immersion oil is used this must also be non-fluorescent.

The elimination of fluorescence from the objective and barrier filters may be difficult and it can be avoided by illuminating the specimen with rays of such obliquity that they exceed the numerical aperture of the objective which one is using. This can be achieved by epi-illuminators or by means of dark-ground illumination. With an aplanatic condenser of N.A. 1·4 separate central stops can be made to suit each objective. A more simple procedure is to use an aplanatic reflecting dark-ground condenser such as the Zeiss cardioid model which has proved very suitable in practice. Its N.A. is 1·22 to 1·38 which means that it transmits less than one quarter of the light transmitted by a bright-ground aplanatic condenser of N.A. 1·4. It has the great advantage that it confines the amount of UV irradiation scattered into the objective to that involved in producing the dark-ground image so that only weak barrier filters are required. Sometimes these may be dispensed with altogether. The dark-ground condenser should have sufficient focal length to produce an adequate field of illumination. The Zeiss cardioid condenser will satisfy an oil immersion or a 4 mm. objective but it cannot be used for objectives of lower powers.

Photography

The image which we require to record is of high contrast and low luminosity and the exposure times are therefore much longer than with ordinary visible light microscopy. The required emulsion is one of high sensitivity and low contrast. For black and white pictures there are many suitable films available. Kodak Super Pan Press Sports or Kodak Super XX were recommended by Price and Schwarz (1956) but Kodak Microfile and Kodak Tri-X have also proved popular. Mayersbach and Pearse (1956) used Kodak Fluorodak green-sensitive, a fast emulsion with suitable sensitivity for the emission wavelengths of fluorescein. Unfortunately the grain size was so large that photographic enlargement was limited to three or four times.

For colour photography daylight films have been found much more suitable than those designed for artificial light. Super Anscochrome or Ektachrome will usually be found satisfactory but Agfacolor can

also be used. Exposures of 15 minutes, or longer, may be required with some of the colour emulsions and stability of the microscope and photographic apparatus, with complete freedom from extraneous vibrations, is thus essential. It is necessary to obtain the correct exposures by trial and error unless a very sensitive light meter is available. Full consideration of photography as applied to fluorescence microscopy is given by Otto (1953) and by Gottschewski and Haase (1953).

Preparations of Sections

The most convenient preparations for observing primary fluorescence, and also for fluorescent antibody studies are freeze-dried or fresh frozen, cold microtome, sections. These are also excellent for fluorochroming procedures (Tobie, 1958). Price and Schwartz (1956) advise, in order of preference, fresh frozen sections, freeze-dried sections and paraffin sections after brief formalin fixation. The latter should be examined, they say, without removal of the paraffin wax. Prolonged formalin fixation increases the bluish autofluorescence of all tissues though it may quench the fluorescence of occasional components such as the lipofuscins. For mounting the sections glycerol and liquid paraffin have been much used in the past but modern media containing polymer plastics are becoming more popular (e.g. *Iso*butyl methacrylate, Dupont ; Fluormount, E. Gurr ; Caedax, Hollborn ; polystyrene in xylene).

Applications of Fluorescence Microscopy

Primary Fluorescence

A great deal of the work in this field has been compressed into the study of a few substances only. Chief among these are Vitamin A (Popper, 1940a and b ; Volk and Popper, 1944), riboflavin (Sjöstrand; 1947), ceroid pigment (Chapter XXIII, p. 666) (Pierangeli *et al.*, 1949 ; Popper *et al.*, 1944 ; Radice and Kaplan, 1950. The porphyrins and the fluorescence of the materials stored in the enterochromaffin cells, have also been extensively studied. Hama and Obika (1958) report a study on the fluorescent pterins of toad skin.

Secondary Fluorescence

Fluorochromes for Nucleic Acids. The most popular of all the fluorochromes used for mammalian and plant tissues is acridine orange (AO) which was introduced for the purpose independently by Bukatsch and Haitinger (1940) and by Strugger (1940). The latter has written a long series of papers culminating (1949) in the publica-

tion of his book on "Fluorescence Microscopy and Microbiology". Strugger was the first to report that AO could be used to distinguish between living and dead protoplasts, the former giving a green and the latter a red fluorescence. Other observers (e.g. Hoefler, 1954) have questioned the accuracy of these observations which are now known, collectively, as the "Strugger Effect".

Acridine orange continues to be used extensively in fluorescence microscopy, especially for the demonstration of the nucleic acids (Plate XA, p. 732) but considerable controversy still reigns over the interpretation of the various findings. Goessner (1949) observed red fluorescing cytoplasmic inclusions in a number of cell types which disappeared after treatment with ribonuclease. He observed that the nuclei in fixed sections also fluoresced red. Hicks and Matthaei (1955) studied extensively the fluorescence of fixed tissues in frozen and paraffin sections stained with AO. In such preparations red fluorescence is hardly, if ever, observed but the nuclei stain a coppery orange colour which contrasts sharply with the yellows and greens of most other tissues. Using either fresh smears or fresh frozen (cold knife) sections von Bertalanffy and Bickis (1956) found that AO staining permitted the identification of red-fluorescent basophilic inclusions in the cytoplasm, removable by ribonuclease, as well as green-fluorescent nuclei. They considered the method a useful one for application to fresh tissues, with or without suitable post-fixation, for the demonstration of RNA. Details of the method are given in Appendix 26, p. 950.

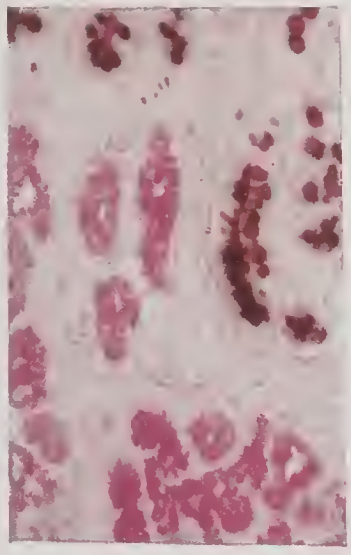
Certain other materials give a red fluorescence with AO in fresh tissues. These are mast cells, cartilage and elastic tissue. In the case of the first two the result is not surprising in view of the ability of acridines to precipitate acid mucopolysaccharides. This feature was made use of by Williams and Jackson (1956) (see Chapter IV, p. 71) in designing their acridine-alcohol fixative for mucopolysaccharides. Examination of tissues fixed in this way by means of fluorescent microscopy does not seem to have been undertaken. Suitable acridines in acetic-ethanol would seem eminently suited to the rapid fixation and demonstration of mucopolysaccharides in fresh frozen sections (diagnostic biopsies).

Acridine orange and the closely related acridine orange-red were used by Armstrong and Niven (1957) in their studies of virus inclusions. They stressed the importance of the pH at which staining is carried out. For AO, on fresh tissues, pH 1·5 to 3·5 gave green DNA and red RNA while for acridine orange-red the pH range to achieve the same results was 3·5 to 5·0. The same technique was applied by Bishop and Austin (1957) to mammalian spermatozoa.

Important studies on the physico-chemical mechanism of AO-

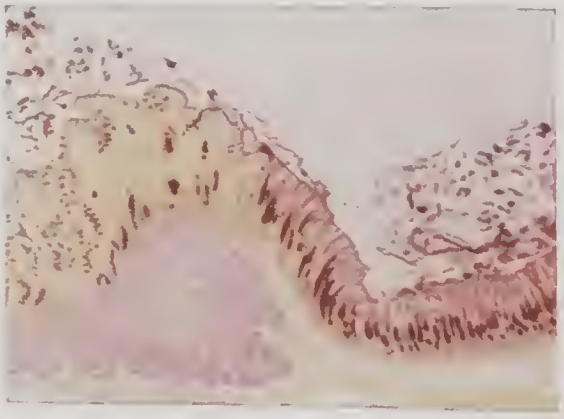
A. Human adrenal gland. Lipid droplets in the cortex fluoresce yellow. Nuclei are copper coloured, connective tissues dull green. Acridine orange. × 145.

XB. Human colon. Bright orange fluorescence of goblet cell and luminal mucins. Iron hæmatoxylin—acridine orange. × 190.



XC. Cat. Brunners glands and bases of crypts of Lieberkühn. Fixed 6 hours after injection of $Na_2{}^{35}SO_4$. Strong radioactivity in goblet cells of the crypts. Exposure 41 days.) PAS. × 120.

XD. Rat. Pylorus and duodenum. Fixed 130 minutes after injection of $Na_2{}^{35}SO_4$. Note strong activity of the pyloric glands. (Exposure 22 days.) PAS. × 20.



XE. Mouse. Colon. Fixed 3½ hours after injection of $Na_2{}^{35}SO_4$. Radioactivity in goblet cells at base of the theca, above the nucleus. (Exposure 14 days.) PAS. × 480.

[*To face p.* 732.

fluorochroming were carried out by Zanker (1952a and b) and the results of these have been largely confirmed by the work of Schümmelfeder, Krogh and Ebschner (1958). Their studies were briefly reported by Schümmelfeder (1958). The tissue employed was Carnoy-fixed mouse cerebellum and the authors agreed with Zanker that the red fluorescence of AO is due to the formation, in stronger solutions, of polymer cations of the dye. They regard the intermediate colours between red and green as due to mixtures of the green monomer and the red polymers. Schümmelfeder and his associates stressed the importance of dye concentration (as others had done previously) as well as pH and they showed that only with very low concentrations (0·01 per cent.) did the cytoplasm and the nucleus stain differently as the pH level was raised from 0·65 to 2·0. With higher concentrations of the dye (0·05 per cent.) the fluorescence of all parts of the cell was green, changing to a uniform yellow with rising pH. Schümmelfeder *et al.* showed, furthermore, that AO in alcoholic solution becomes bound to nucleic acid in two ways : (1) by electrostatic (salt) linkages and (2) by cohesive (van der Waals) forces. They found that RNA (Nissl substance) bound AO progressively up to pH 6·0 whereas with the DNA of the nuclei the amount of dye bound increased only up to pH 3·0. After depolymerization, however, DNA was observed to behave towards AO in the same way as RNA. These findings have an obvious parallel in the behaviour of the nucleic acids in the methyl green-pyronin method (see Chapter VIII, p. 205).

Distinction between nucleic acids and mucopolysaccharides, which tend to bind AO at lower pH levels than the nucleic acids, is best made in sections treated first with ribonuclease.

Fluorochromes for Mucins. In addition to the above-mentioned staining of mucopolysaccharides in fresh sections by means of AO an interesting modification has been described by Hicks and Matthaei (1958). These authors observed that if AO was applied to tissues after Masson's trichrome stain the epithelial mucins were apparently specifically stained so that they produced a bright orange-yellow fluorescence (Plate XB, p. 732). This effect was found to be due to the mordant. Pretreatment with either ferric chloride or iron alum was found to quench most of the normal AO fluorescence of the tissues but subsequent overstaining with AO produced the " specific " staining of mucins. This method may be found useful and details are therefore given in Appendix 26. In any case it is an interesting example of the application of the principle of differential quenching. Fluorescence techniques for mucins have also been described by Kuyper (1957).

Fluorochrome for Lipids. A number of these have already been described in Chapter XI, p. 301, and two methods are given in Appendix 11.

Fluorescent End-Point Methods

Fluorochromes for Inorganic Components. Several fluorescent end-point methods for aluminium and calcium have been described in Chapter XXIV. Other end-point methods are also described in the text, such as the fluorescent HNI method for SH groups (Chapter V, p. 118). Extension of this principle may confidently be expected.

Fluorescent Tracers

The compounds described in this section can be, or have been, applied to the problem of tracing the fate of injected foreign protein antigens. They are equally applicable to labelling of antibodies for use in the fluorescent antibody methods (Chapter VI, p. 137) and the particular compounds discussed below are dealt with in this chapter, rather than in Chapter VI, mainly because they have been developed since that chapter was written.

The following requirements, which should be satisfied by fluorescent compounds designed for use in tracer experiments. have been set forth by Chadwick, McEntegart and Nairn (1958b). Such compounds should :

(1) Possess chemical groups capable of reacting directly with proteins to form a stable linkage such as azo, carbamido or sulphonamido.
(2) Not possess groups capable of interfering with the reaction with protein.
(3) Have an intensity of fluorescence (fluorescence yield) of the same order as fluorescein (see Chapter VI, p. 146) and this should not be diminished appreciably by conjugation.
(4) Give a fluorescence colour of longer wavelength than fluorescein so as to provide better contrast.

To these requirements one might add :

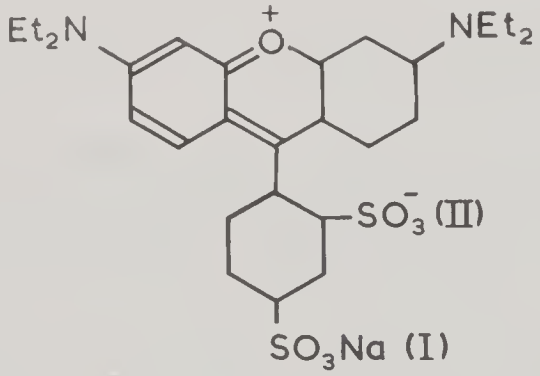
(5) That the compound should be stable and that its solubility in water, and its reactivity, should be such that is can be used for conjugation under conditions leading to the minimum amount of denaturation of protein.

Alternatives to Fluorescein *Iso*cyanate

Since fluorescein *iso*cyanate is difficult to prepare, has a low solubility in water, and only moderate reactivity, many attempts have been made to find suitable substitutes. The first compound to be used as an alternative fluorescent label was 1-dimethylaminonaphthalene-5-sulphonyl chloride (DANSYL) which has been discussed extensively in Chapter VI. The effect of this compound on antigen-antibody

reactions was tested by Redetski (1958) who found that γ-globulin-DANSYL conjugates contained 3–4 molecules of dye per mole of protein and that this amount of labelling had no effect on the formation of an antigen-antibody complex. Unfortunately, measurements of remaining free dye in the supernatant after precipitation of the protein conjugate do not take into account the amount of adsorbed dye. Redetski's statement that precipitation with alcohol or acetone does not split the protein-DANSYL "bond" must be regarded with caution until proof is offered that such a chemical bond is in fact formed under the usual conditions of conjugation.

Lissamine Rhodamine Sulphonyl Chloride. This compound was introduced for fluorescent labelling by Chadwick, McEntegart and Nairn (1958a and b). The formula for the parent dye, lissamine rhodamine RB200 (I.C.I. Ltd.) is shown below.

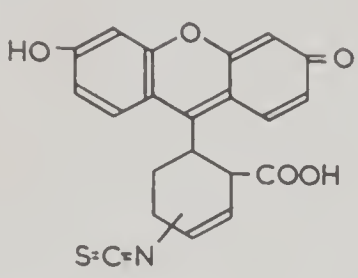


This dye, as can be seen, has two sulphonic acid groups available for conversion to sulphonyl chlorides. Under mild conditions (e.g. chlorosulphonic acid) it is probable that only one of these (I) will be converted, giving a product insoluble in water. Under more severe conditions (PCl_5) both groups (I and II) undergo conversion to sulphonyl chlorides giving a basic dye which is soluble in water but rather easily decomposed. Directions for the synthesis of lissamine rhodamine disulphonyl chloride are given in Appendix 6, p. 813.

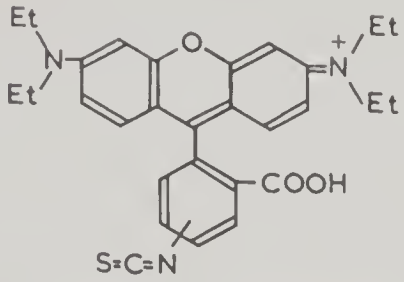
This compound has been successfully employed by Chadwick *et al.* for the labelling of proteins for tracer and antigen-antibody methods. Its fluorescence when irradiated with UV light is a deep orange-red and this provides an excellent contrast with the autofluorescence colours of various tissue components. Fading is rapid, however, especially if blue light is used for irradiation of the object.

Rhodamine B Isocyanate. This compound, which fluoresces orange-red, was synthesized by Silverstein (1957) and used by Silverstein, Eveland and Marshall (1957), chiefly in order to provide a contrasting label to fluorescein *iso*cyanate. It suffers, presumably, from the same deficiencies as the latter, which have led to the present intensive and widespread search for suitable substitutes.

Fluorescein Isothiocyanate and Rhodamine B Isothiocyanate. Recently Riggs *et al.* (1958) have prepared the stable solid *iso*thiocyanates of both fluorescein and rhodamine B and these, especially the former, appear to overcome most if not all of the technical difficulties attached to fluorescein *iso*cyanate. The formulæ of the two compounds are given below :



Fluorescein *iso*thiocyanate



Rhodamine B *iso*thiocyanate

Details of the preparation of these two *iso*thiocyanates are given in Appendix 26.

Evidence for the superiority of fluorescein *iso*thiocyanate over fluorescein *iso*cyanate has been provided by the work of Marshall, Eveland and Smith (1958) who have compared the fluorescent staining titres of the two compounds. They found much higher titres with the *iso*thiocyanate and correspondingly more intense staining was obtained. Conjugated antisera to *Pasteurella tularensis* could be used in 1 : 160 dilution whereas the *iso*cyanate-conjugated sera would not give a positive result in dilution greater than 1 : 20.

Since the *iso*thiocyanate is a stable powder which can be added directly to the cold buffered serum its technical advantages make it certain that it will replace fluorescein *iso*cyanate for tracer and antigen-antibody studies. Nevertheless this compound, and all others used as fluorescent labels, require rigorous testing in respect of their ability to form chemical linkages with the amino groups of the conjugated protein.

Cellulose Reactive Dyes. A large number of reactive groups are known which will combine rapidly with the hydroxyl groups of cellulose fibres at alkaline pH levels to form stable covalent bonds. Under similar conditions these groups can react with the free amino or hydroxyl groups of proteins. These reactive groups include thiocyano, mono and dichloro-triazinyl, sulphon-β-chloro ethylamido, sulphonfluoride, chloroacetylamino, and γ-chloro-β-hydroxypropyl.

A series of coloured or fluorescent compounds containing these groups was tested by Hess and Pearse (1958) in tracer protein experiments and the fluorescent compounds were also tested as labels in antigen-antibody reactions. In these experiments the best results

FIG. 232. Mouse kidney. Fresh frozen section. Animal given intraperitoneal injection of ovalbumin labelled with Procion black HBS, 36 hours previously. Intracellular deposits of the labelled protein can be seen. × 1,400.

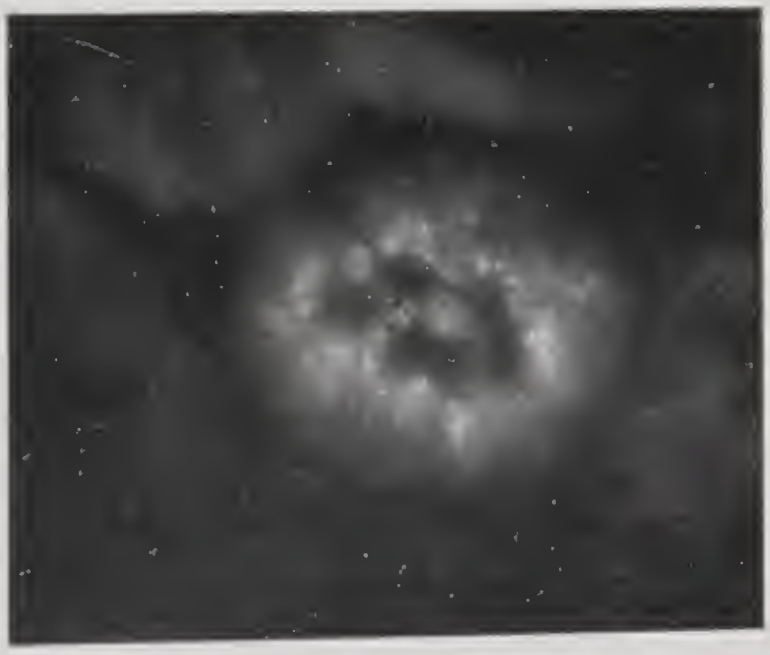
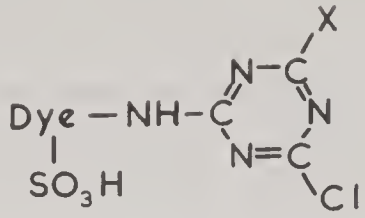


FIG. 233. As Fig. 232 but animal injected with ovalbumin labelled with a fluorescent dichlorotriazene dye, 20 hours previously. Small intracellular protein deposits are brightly fluorescent. × 1,400.

[*To face p.* 737.

were obtained with dyes (coloured or fluorescent) possessing the mono or dichloro-triazinyl group.



In the above formula Dye-SO_3H represents the sulphonated chromophor (azo, anthraquinone, phthalocyanin, etc.) and x stands for either a chlorine atom or an inactive radical.

The fact that firm chemical linkage results from the conjugation of proteins with mono or dichloro-triazinyl compounds was demonstrated by a method originally described by Vickerstaff (1958). A yellow reactive dye possessing an azo chromophor was coupled to ovalbumin. The dye-protein compound was then reduced with sodium hydrosulphite to a colourless product containing free amino groups (from the split azo linkage). This was then diazotized and coupled with naphthol AS to give a deep red protein-azo dye.

Some of the more highly coloured reactive dyes containing the dichloro-triazine grouping were used to label proteins in tracer experiments in mice. The result given by coupling the blue dye Procion blue HBS (I.C.I. Ltd.) with ovalbumin are shown in Fig. 232 where the protein droplets in the renal tubules are demonstrated 18 hours after injection of the labelled conjugate. Better results were obtained, however, by using compounds containing a fluorescent chromophor and these are illustrated in Fig. 233, using the same test system described above.

When reactive dyes are coupled with whole serum the result, as with all the fluorescent labelling compounds, is a preferential attachment of the dye to the albumin fraction. Labelling of the γ-globulin fraction may be minimal. If a purified γ-globulin fraction is used, however, labelling with coloured or fluorescent dichloro-triazinyl compounds is quite satisfactory and relatively large amounts of dye can be coupled to the protein if required. In experiments with Procion blue HBS as much as 50 mg. of dye was coupled to 50 mg. of serum albumin (20° ; pH 10·0 ; 1 hour) with recovery, after dialysis, of 2 per cent. of the original weight of dye. It is difficult to determine precisely the amount of dye chemically linked to the protein and the amount which is physically adsorbed. It is probable, however, that with most of the compounds at present used for fluorescence labelling (except the dichloro-triazines) physically adsorbed dye exceeds chemically bound dye by a considerable margin. The dangerous consequences of this in fluorescent tracer or antigen-antibody studies

are sufficiently obvious (see Chapter VI). It is essential that critical studies be performed as soon as possible to measure the precise reactivity of all the compounds currently used in immunohistological techniques. Comparisons at present are based only on technical convenience and on the final effect produced in tissue sections. Studies like those of Redetski (1958) and of Marshall *et al.* (1958), though valuable, have not attempted to tackle the hard core of the problem.

Foreign Substances

A list of fluorescent compounds introduced into the tissues for therapeutic or other purposes, and studied thereafter by fluorescence microscopy is not a large one. Reference has already been made in Chapter XXV to the demonstration of quinacrine (atebrin) in human skin by Mustakallio (1954), whose studies were based on earlier animal experiments by Jailer (1945). The distribution of this substance in *Tænia saginata* was studied by Mustakallio and Saikkonen (1954) after its (successful) use as a vermifuge.

The fluorescence of mouse skin, after painting with benzpyrene in acetone or benzene, was examined by Beck and Peacock (1940), by Hamperl, Graffi and Langer (1942), and also by Doniach, Mottram and Weigert (1943). Simpson and Cramer (1945) observed a blue fluorescence after the application of methylcholanthrene to the skin. The problem of benzpyrene and its rate of disappearance from mouse skin after a single application was extensively studied by Nordén (1953).

A fluorescent pteridine compound (2-methylamino-4-amino-6, 7-diphenylpteridine) which has a selective action on myocardial muscle has been described by Paget (1957). This can be detected in the tissues by fluorescence microscopy. Antibiotics such as dihydrostreptomycin have been followed by this means, after absorption (Abello, 1954) and Alin and Helander (1948) used the method to detect the distribution of *p*-amino-salicylic acid. Flavin compounds are readily studied by fluorescence microscopy and the trypanocidal drug trypaflavin was investigated in this way by Jancsó (1932). Many further examples of the tracing of foreign substances are to be found in the comprehensive reference list published by Reichert (1956).

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CHAPTER XXVII

AUTORADIOGRAPHY AND ITS APPLICATIONS

Autoradiography

The basis of autoradiography or radioautography is the demonstration of radioactive isotopes in tissue sections by means of their ability to reduce silver salts in a photographic plate or emulsion. The method is by no means a new one, having been employed as early as 1904 by Loudon. Kotzareff (1922) and Lacassagne and Lattes (1924) carried out autoradiographic studies, the latter authors using polonium in paraffin-embedded material. At a later date successful studies with radioactive lead in bones were made by Behrens and Baumann (1933a and b), and with radioactive phosphorus by Dols *et al.* (1938). In the case of radioactive iodine the studies of Hamilton *et al.* (1940) on the thyroid gland are particularly important from the historical point of view. Pecher (1942) used phosphorus for studies of bone and soft tissues and strontium in the case of bone tumours. Treadwell *et al.* (1942) also used strontium in a similar manner.

Since 1940 the techniques and practice of autoradiography have increased considerably in scope and achievement. A large number of radioactive elements have been used and these fall naturally into two main groups.

Substances Foreign to the Tissues. Included here are polonium and strontium, already mentioned, and other elements such as gallium, radium and gold. Strontium and radium, when administered in ionic form, mostly end up in the growing ends of bone and gallium (Dudley and Maddox, 1949) has been found to have a similar distribution. Endicott and Yagoda (1947) injected polonium (Radium F) in buffered physiological saline into mice. Radioactivity was localized especially in the spleen and in the renal glomeruli. Besides these elements, administered in ionic form, a number of others have been given in combined form in substances foreign to the tissues. Axelrod and Hamilton (1947) studied the distribution of sulphur-labelled mustard gas and arsenic-labelled lewisite in the tissues of the skin and eye.

Physiological Substances. In the case of substances occurring naturally in the tissues studies have been made either with the elements in ionic form or with various combinations into organic molecules. Bloom *et al.* (1947) injected rats with ^{14}C-labelled bicarbonate and found marked deposition in the shafts of the long bones and in liver and kidney. Armstrong *et al.* (1948) made similar investigations of

^{14}C distribution in the rat. Further details were given by Bloom (1949). Boyd *et al.* (1948) described the localization of ^{14}C in individual blood cells, and Boyd and Levi (1950) were able to show β-particle tracks from the livers of rats injected with ^{14}C-labelled glycine. Numerous authors have made studies with ^{32}P (e.g. Bélanger and Leblond, 1946 ; Percival and Leblond, 1948 ; Palm, 1948 ; Bulliard *et al.*, 1938 ; Marshak, 1941 ; Bayley, 1947) and these have demonstrated a number of interesting points. Radioactive phosphate ions exchange with non-radioactive phosphate in a large number of situations, but most rapidly, apparently, where there is a rapid rate of metabolism. They have been shown to be incorporated both into phospholipids, and also into the nucleic acids (Leblond, Stevens and Bogoroch, 1948). These authors considered that normally all ^{32}P introduced into the tissues was incorporated into newly-formed nucleic acids.

Dudley and Dobyns (1949) illustrated the deposition of radio-calcium in the subperiosteal region of ribs and Berggren (1946) studied the distribution of ^{24}Na in teeth. This element has been studied in ocular tissues by von Sallman *et al.* (1949). Radiopotassium was shown by Colfer and Essex (1946) to be fairly uniformly distributed through the brain of the rat, with a tendency to greatest concentration in the cerebral and cerebellar cortex, and in the basal ganglia. Howard and Pele (1951) treated seedlings with ^{32}P and subsequently extracted the root tissues with hot HCl. After this treatment ^{32}P was present in the form of DNA-phosphorus. Using this method the authors found that DNA synthesis occurs in early interphase only, in those cells which are preparing for division. When ^{35}S was used in place of ^{32}P Howard and Pele found a positive result in the same proportion of nuclei as gave a positive result with ^{32}P. They interpreted this finding as indicating the build-up of sulphur-containing amino-acids in parallel with nucleic acid synthesis. Figs. 234 and 235 illustrate the results obtained in a single chromosome from a root meristem squash.

Radio-iodine (^{131}I) has been more extensively used than any other element and this is largely due to the ease with which it becomes incorporated into thyroglobulin after being initially concentrated in the thyroid gland in inorganic form. The majority of thyroid auto-radiographs (ARG) illustrated in the literature show protein-bound ^{131}I mainly confined to the colloid. Leblond and Gross (1948), however, maintained that in iodine-deficient rats incorporation of ^{131}I took place mainly in the luminal part of the lining cells of the acinus. Since all inorganic ^{131}I is removed during the processing of the tissues, this was considered to indicate the production and secretion into the acinus of new thyroglobulin. Doniach and Pele (1949) were unable to confirm these findings and they considered that protein-bound ^{131}I was always situated in the colloid. These authors (Doniach *et al.*, 1952) suggested

that ^{131}I diffused through the acinar cells and was bound to colloid within the follicle lumen. The iodination of colloid, and the eventual formation of thyroxine-thyroglobulin, depend on the amount of iodine and thyroid-stimulating hormone present in the follicle and also on the follicular cell enzymes. According to Doniach *et al.*, the two types of thyroid ARG (*Ring* and *Blob*) may represent two different types of hormone synthesis in the underactive and normally active follicles respectively.

Other interesting studies of thyroid function have been made with the assistance of ^{131}I. Gorbman and Evans (1941) prepared ARG in the developing thyroids of tadpoles kept in water containing ^{131}I. They were able by this means to show that storage of iodine commenced as soon as follicles appeared in the developing thyroid gland. Later Gorbman (1947) demonstrated by means of ARG the presence in mouse thyroids of non-functional structures which he considered to be derived from the ultimobranchial bodies. The use of ^{131}I is not confined to studies of thyroid function. Albert *et al.* (1949) used radioactive iodo-alpha-œstradiol (non-biologically active) and found that after injection this substance was localized in the dermal connective tissues and, to a lesser extent, in liver, intestine and breast.

By the incorporation of radioactive elements into organic substances much interesting work has been produced. Boyd *et al.* (1948) used glycine labelled with ^{14}C and showed that this substance became incorporated into liver cells, white blood cells, and into a small proportion of the circulating erythrocytes. Proteins have been labelled by combination with various radioactive elements, the most popular, because it is the easiest to apply, being ^{131}I. This element is taken up by the tyrosine groups of the protein and the number so iodinated is subject to fairly accurate control. If only a small percentage of the available tyrosine is iodinated the properties of the original protein are not radically altered. Using such a method Warren and Dixon (1948) showed that in the guinea-pig killed by anaphylactic shock due to sensitivity to bovine gamma globulin, the antigen was concentrated not in the smooth muscle of the bronchi but in the peribronchial connective tissues. Pressman *et al.* (1949) showed by a similar means that anti-mouse kidney serum globulin, labelled with ^{131}I, could be found after injection solely in the glomeruli as far as the kidney was concerned.

Hormones labelled with ^{131}I have been used by several authors for the demonstration of the supposed sites of specific activity. Jensen and Clark (1951) administered ^{131}I-labelled thyroxine to rabbits which they killed four hours after the injection. Strong ARG were obtained from sections of the neurohypophysis and tuber cinereum. Sonenberg *et al.* (1951a) showed that ^{131}I-labelled ACTH gave positive ARG

only in adrenals and thyroid and this same author (Sonenberg *et al.*, 1951b) demonstrated that labelled prolactin gave no significant ARG in resting or lactating mammary glands. In the ovary, however, strong ARG were obtained and these were confined to the corpora lutea.

The use of ^{131}I-labelled iodate in the iodate reaction for the demonstration of noradrenalin in the adrenal medulla (Eränkö, 1957) is shown in Fig. 237. The presence of iodine in the noradrenochrome pigment is clearly indicated.

Recent Applications

The greatest volume of studies continues to be in the nucleic acid field and a variety of labelled substances have been employed. Considerable improvement in localization has been produced by the substitution of ^{14}C-labelled adenine for ^{32}P as phosphate. Clowes (1956) used the former, together with the stripping film technique, in studying the metabolism of nucleic acids in *Zea mays* seedlings. Pelc and his associates (Pelc, 1958 ; Hornsey and Howard, 1956 ; Pelc and Howard, 1956 ; Pelc, 1956 ; Lasnitsky and Pelc, 1956) have continued their studies in this field, using the same methods. Electron track autoradiographs of sections prepared by the coating technique were used by Ficq (1955) to obtain intracellular localization of ^{14}C-adenine in frogs and Ficq and Errera (1958) have studied the metabolism of phenylalanine-2-^{14}C in isolated nuclei. Walker and Leblond (1958) used ^{14}C-adenine and ^{14}C-thymidine to demonstrate sites of nucleic acid synthesis in the mouse. Following treatment of the sections with ribonuclease sites of incorporation of ^{14}C-adenine were interpreted as sites of newly synthesized DNA. The chief sites of production were the epithelia of the digestive tract and other epithelia, and the germinal centres of the lymph nodes and spleen. Considerable activity was also observed in many types of connective tissue.

Tritium (^{3}H) was introduced as a radioactive label by Fitzgerald *et al.* (1951) and this isotope has been used recently on an increasing scale. Tritium-labelled adenine and thymidine, according to McQuade *et al.* (1955), is incorporated solely into DNA and by the use of labelled thymidine, therefore, selective extraction procedures can be avoided (Plaut and Mazia, 1956).

A large amount of work continues to be done with the aid of ^{35}S, particularly in the use of inorganic ^{35}S and ^{35}S-methionine in studies of the metabolism of sulphur in the acid mucopolysaccharides (Boström and Odeblad, 1953, 1954 ; Bélanger, 1953, 1954 ; Davies and Young, 1954 ; Engfeldt *et al.*, 1954 ; Friberg and Ringertz, 1954 ; Boström *et al.*, 1954 ; Fajberg and Ringertz, 1954 ; Pelc and Glucksmann,

FIG. 234. Isolated chromosomes in a root meristem squash (20 hours, S^{35}). Stripping-film autoradiograph. × 2,000.

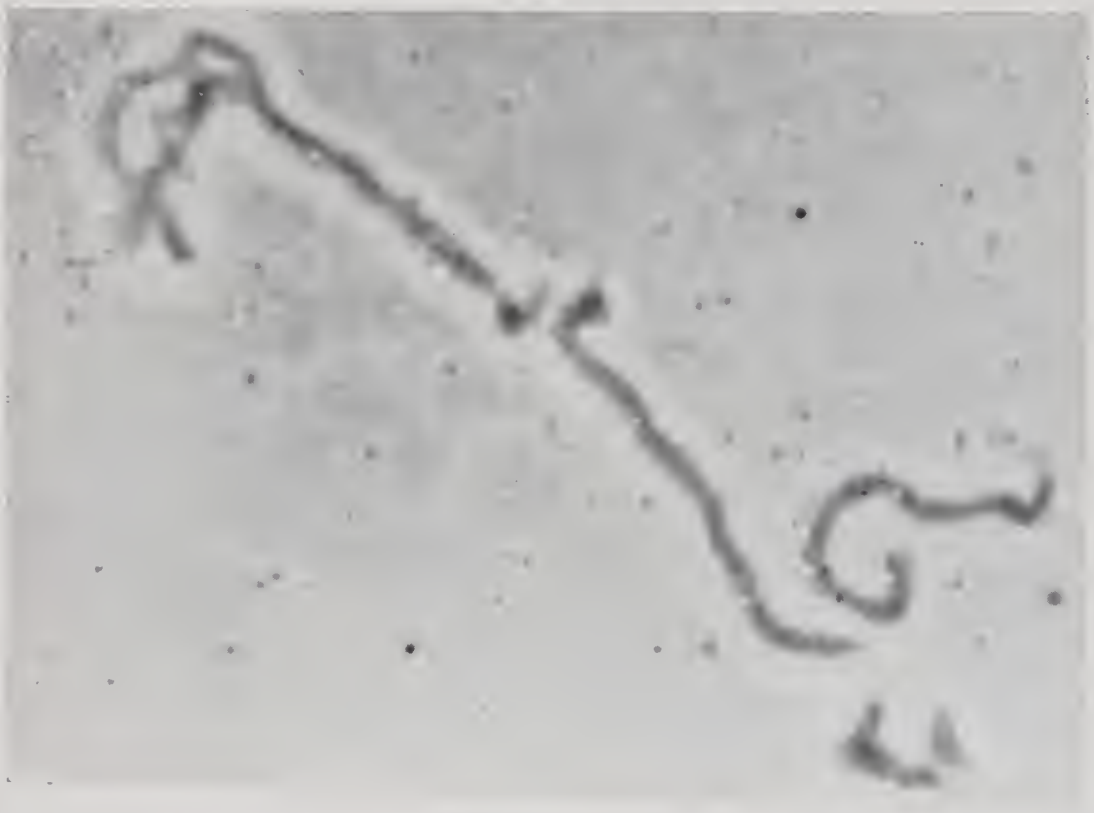

FIG. 235. As Fig. 234, with addition phase contrast. To show localization of the silver granules in the film in relation to the chromosomes. × 2,000.

[*To face p.* 744.

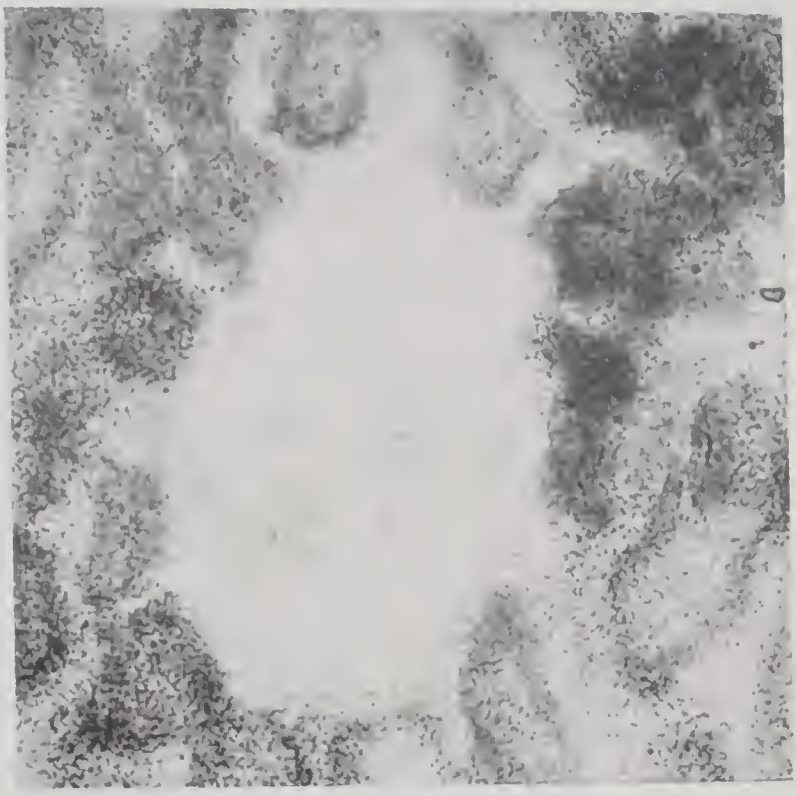

FIG. 236. Rat kidney. Succinate dehydrogenase reaction using ^{58}Co in standard MTT-cobalt method. Autoradiograph. × 360.

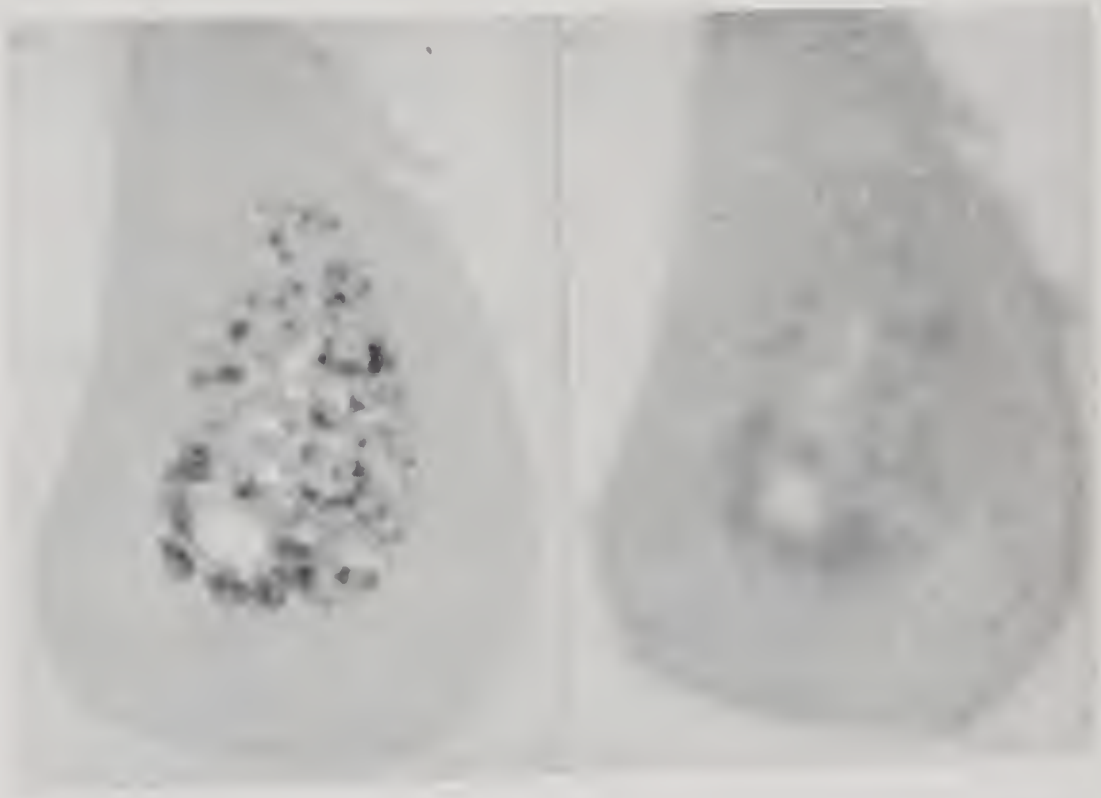

FIG. 237. Mouse adrenal gland. Iodate reaction (*left*) using ^{131}I—labelled iodate shows brown pigment in noradrenalin-containing cells. Autoradiograph (*right*) of same section shows that iodine is present in the pigment. × 110.

1955 ; Fitzgerald *et al.*, 1954 ; Dziewiatkowski, 1956 ; Johnson and Comar, 1957 ; Gracheva, 1957 ; Lea and Vaughan, 1957). An extensive study on the epithelial mucins in 5 species was made by Jennings and Florey (1956). These authors studied the uptake of ^{35}S, administered intravenously either as $Na_2\,^{35}SO_4$ or as ^{35}S-methionine, and they found that with the latter radioactivity was situated diffusely throughout the gastrointestinal tract without being concentrated in any particular area. After $Na_2\,^{35}SO_4$, however, they noted that certain epithelial cells of the stomach and duodenum took up the radioactive label and incorporated it into their mucopolysaccharides. In the rat (Plate Xc, p. 732) the pyloric glands were strongly active while the Brunner's glands of the duodenum were inactive. In the guinea-pig almost the opposite condition was observed. In the cat (Plate Xd, p. 732) the strongest activity was found in the goblet cells in the crypts of Lieberkühn.

Lower down in the intestinal tract the incorporated ^{35}S was found largely in the goblet cells at the bases of the crypts and with these cells (Plate Xe, p. 732) radioactivity was concentrated at the base of the theca, above the nucleus.

Metallic Ions. Comparatively few studies have been carried out using isotopes other than the more or less conventional ones although many are quite suitable for autoradiographic studies (Taylor, 1956). Among these are ^{45}Ca, ^{22}Na, ^{52}Mn, ^{54}Mn, ^{65}Zn, ^{42}K, ^{58}Co and ^{59}Fe. Radioactive calcium was used by Bélanger *et al.* (1954) in autoradiographic studies of the growth of incisor teeth in fluoride-treated pigs, and the uptake of ^{45}Ca by bone slices *in vitro* was reported by Amprino (1952). Radiopotassium was used by Morel and Guinnebault (1956) to study the pattern of tubular excretion in the rabbit kidney. Most of the specific activity was found in the cortex, with a very high exchange rate (15 per cent. per minute).

Studies with radioactive zinc were made by Lowry *et al.* (1954) but these did not include autoradiography. The use of ^{65}Zn was also mentioned by Steinberg and Solomon (1949) and ^{59}Fe was employed in autoradiographic studies by Austoni (1954).

A rather different principle in applied autoradiography was used by Pearse (1958). In this case the standard incubation medium for the dehydrogenase techniques (Chapter XX and Appendix 20) was modified by the inclusion of a small proportion of ^{58}Co in addition to the usual non-radioactive isotope. The result is shown in Fig. 236. It was hoped that quantitative autoradiography (see below) of the ^{58}Co-formazan would allow estimation of the activity per cell of the various dehydrogenases. Interference by non-specific absorption, however, prevented the realization of this aim.

The use of ^{212}Pb by Barka *et al.* (1952) as a radioactive capture

reagent for phosphate ions in the alkaline phosphatase technique has already been mentioned in Chapter XIV, p. 400.

The Technique of Autoradiography

Physical Considerations. It is not proposed to enter thoroughly into the physical aspects of autoradiography in this chapter. For theoretical considerations of these the reader is referred to excellent reviews on the subject by Heller, Axelrod and Hamilton (1950), by Gross *et al.* (1951), Doniach *et al.* (1952), and by Fitzgerald (1955), Taylor (1956) and Pelc (1958). Many theoretical aspects were discussed also by Doniach and Pelc (1950). The points dealt with by all the above authors include the relationship between the source of radioactivity in the tissue and the overlying emulsion, the mechanism of photographic action in the emulsion, the dose and energy of the radiation employed, the characteristics of the emulsion and the conditions of its development and fixation.

Summarizing briefly, it appears that the finest resolution is obtained by the reduction of the thickness of the section, and of the width of the interspace between the section and the overlying emulsion, to a minimum. The maximum theoretical resolution with a 2 μ section, a 2 μ emulsion, and a 0·1 μ interspace has been calculated to be 2·3 μ. Figs. 234 and 235 indicate that the resolution obtained in practice is very near this figure. Resolution is decreased by prolonged exposures because the diameter of the image is thereby increased. As far as the emulsion is concerned, the larger the grain size the more β-particles are trapped and the greater the amount of energy absorbed. Emissions from radioactive material are of three types, alpha, beta and gamma particles, but the majority of isotopes normally employed are beta and gamma emitters. The greater the grain size, the greater the number of silver atoms reduced by the same amount of radiation and, therefore, the larger the grain the greater the apparent sensitivity of the emulsion when examined by the low-power microscope. At the same time the resolving power is much lower (owing to the larger image). For this reason, from the point of view of accurate localization, the use of fine grain emulsions is preferable. Some improvement in sensitivity can be achieved in these fine grain emulsions by increasing the concentration of grains to a maximum, and by sensitizing the grains in various ways.

Histological Considerations. Any histologist who intends to apply the technique of autoradiography will presumably obtain sufficient advice on the handling of radio-isotopes and, on their administration if this is involved, from those who supply him with his materials. If he is called on, however, to make an ARG of material handled entirely by others in its earlier stages, as may well be the case when isotopes

are being used therapeutically, it is essential that he should possess some knowledge of the various methods of processing. Only the processing of soft tissues will be considered here ; details of the preparation of bone and teeth for autoradiography will be found in papers by Leblond *et al.* (1950), Laude *et al.* (1949) and Sognnaes *et al.* (1949).

Although it is possible to make ARG of unfixed thick, frozen sections by the contact method (see below), for most purposes autoradiography involves the preparation of thin slices of fixed tissue. The choice of fixative to some extent depends on the nature of the isotope concerned and on the tissue component with which it has become incorporated if this has occurred. Protein-bound isotopes have been studied in material fixed by most of the conventional fixatives. Endicott and Yagoda (1947) used formalin and Bélanger and Leblond (1946) stated that commonly used fixatives such as Bouin and neutral formalin did not interfere with the technique. Evans (1947) used fixation, dehydration, embedding and sectioning " according to the usual histologic techniques." Doniach and Pelc (1950), however, tried formal saline, Bouin, Helly, Susa and absolute alcohol as fixatives for rat thyroids containing protein-bound ^{131}I. They found, with the first two fixatives, a peculiar patchy inhibition of the photographic process whereby parts of the film, on subsequent development, appeared devoid of silver granules. The two mercury-containing fixatives also produced an occasional inhibition and sections fixed in them suffered from the disadvantage that mercury deposits could not be removed in the usual manner with iodine and thiosulphate, in case this treatment caused removal of ^{131}I also. Doniach and Pelc therefore advised fixation in absolute alcohol until such time as the process of autoradiography allowed fine cytological localizations to be made. Control experiments, using non-radioactive sections, were invariably found to be essential.

In the case of water-soluble isotopes a somewhat different technique is necessary. Holt *et al.* (1949) found considerable loss of ^{32}P from formalin-fixed paraffin sections floated out on water. They tested three methods in particular for avoiding this loss : (1) Freeze-dried sections embedded in paraffin. (2) Alcohol-formalin fixation (2 hours), followed by dehydration in alcohol, clearing in xylene and paraffin embedding. (3) Alcohol-formalin fixation followed by dehydration and clearing with dioxane, and paraffin embedding. Floating out was avoided in each case, and the third method was found to be most satisfactory for avoiding loss of water-soluble ^{32}P. They also froze small pieces of tissue at — 170° and then immersed them in absolute alcohol saturated with basic lead acetate, in order to precipitate phosphate as the lead salt. MacDonald *et al.* (1949) used a method for preparing frozen sections originally described by Linderstrøm-Lang

and Mogensen (1938). This consisted of freezing the tissues in liquid nitrogen and sectioning on a freezing microtome in a cold room at — 10° to — 15°. The sections were then melted on to slides, covered by stripped emulsion, and exposed and developed before staining. Good preservation of water-soluble ^{32}P was obtained. Cold microtome sections, mounted on coverslips or half slides by the use of a suction pick-up (Appendix 2, p. 784) are ideal for autoradiographic studies. Contact with water is easily avoided but water-soluble isotopes are still best dealt with by the use of freeze-dried sections. Harris *et al.* (1950) used the standard freeze-drying process, but flattened their sections on warm mercury to avoid contact with water.

On the whole, provided that only protein-bound radio-isotopes are being studied, fixation in absolute alcohol followed by brief clearing in benzene and by paraffin embedding, will be found to give reliable and satisfactory results.

Three Basic Methods. *Contact Autoradiographs.* The simplest of the three methods of autoradiography is the contact method. This requires no special equipment and can easily be performed in any routine laboratory. It depends on the placing of the tissue slice or section in contact with a photographic plate and maintaining contact for an appropriate period. The results with this method are poor in that resolution is bad (50–100 μ), but the speed obtained is high if fast X-ray film is used. Dobyns and Lennon (1948) used contact ARG to determine the presence of active and inactive nodules in operative specimens of human thyroids and they obtained much useful information by this means.

Mounted Autoradiographs. This method, first described by Endicott and Yagoda (1947) and later by Evans (1947), depends on the mounting of sections (5 μ thick in the original article) directly on to the photographic emulsion. Staining may be carried out before or after mounting, exposure and development.

Coated Autoradiographs. There are three types of coated ARG : in the first, described by Bélanger and Leblond (1946) and by Leblond, Perceval and Gross (1948), sections mounted on slides were covered by a thin layer of photographic emulsion. Originally the sections were unstained, but later they were stained before treatment with the emulsion. The second type of coated ARG is the stripping film type described by Pele (1947) and later by Doniach and Pele (1950). In this type of ARG a layer of emulsion, on its gelatin base, is stripped from the glass of the photographic plate and placed, with the emulsion downwards, over the mounted section. Staining is sometimes carried out before application of the film but objections have been made to this procedure on account of possible displacements of radioactive materials which may take place. Alternatively (Doniach and Pele,

1950 ; Simmel *et al.*, 1951), the preparation may be stained through the gelatin base after exposure and development. Some of the newer plastic bases are impermeable to the developer and must be placed on the sections emulsion side upwards. This results in poorer resolution owing to the increased width of the interspace and post-staining is impossible. The third type of coated autoradiograph is the so-called inversion type, introduced by MacDonald *et al.* (1948) and by Bélanger (1950). With this procedure the mounted unstained tissue section, covered with celloidin, is coated with a special emulsion (Kodak matrix). After exposure and development, the preparation is immersed in water and the emulsion is lifted from the slide with a razor blade, the section adhering to its undersurface. It is then inverted and mounted, section upwards, on a clean slide. Staining can now be carried out with ease.

In this country the most popular technique is that of Pelc, using stripping film. Since this method is capable of giving resolution down to 2 μ, and since it is also capable of giving satisfactory results with a considerable degree of regularity, technical details are given in Appendix 27. Details of a stripping film technique, and also of the other techniques mentioned above, are to be found in the review of Gross *et al.* (1951). Because of the usefulness of the contact process where fine resolution is not required, details of this method are also given in Appendix 27.

Technical Considerations of the Stripping Film Method. With the older stripping film techniques some trouble was experienced on account of displacement of the photographic image by movement of the film. This displacement was of two types : (*a*) generalized and (*b*) localized. Generalized displacement occurred when the whole emulsion and gelatin backing became shifted relative to the underlying section and it was overcome by paying particular attention to efficient attachment of the emulsion to the slide. Bogoroch (1951) suggested the wrapping of the base and emulsion completely round the slide and emphasized the importance of sufficient preliminary soaking. For this purpose she advised a Dupanol-water mixture (Dupanol C, or lauryl sulphate) and 5 minutes immersion of the emulsion before its application to the slide. After application the preparations were thoroughly dried in a desiccator over $CaCl_2$. Localized displacement occurred with folding and swelling of the emulsion and this was overcome by efficient soaking of the type described above. Coating of slides with a film of gelatin previous to mounting of the sections (Doniach and Pelc, 1950 ; Berriman *et al.*, 1950) is an efficient method of avoiding both generalized and localized displacement but, in practice, it will be found that displacement of both types is entirely avoided by strict adherence to the coating and processing temperatures recom-

mended in Appendix 27. No displacement is to be expected during the processes of staining and mounting, but it is advisable to dehydrate, clear and mount the section, soaking in dilute mountant for 30 minutes before using the full strength, and to leave it until the mounting medium has hardened, before trimming off the redundant emulsion. If any lifting of the emulsion is observed on arrival at the stage of dehydration, it is preferable to mount the section in an aqueous medium of the glycerine jelly type.

There remain some brief considerations of technique which are common to all the methods described above. The *duration of exposure* must be kept to a minimum since over-exposure, by producing a diffuse image, reduces resolution considerably. Theoretically, the time of exposure can be calculated by estimating the amount of radioactivity in the sections by means of a Geiger counter. In practice, however, it is usual to employ a series of sections and to develop these at intervals until the optimum ARG is obtained. Some authors advise that exposure should be carried out at 4° or even at — 10°, especially for long exposures, in order to reduce fogging to a minimum. For *localization of radioactivity*, phase contrast microscopy has proved especially useful. By this means it is possible to visualize structural details of unstained material and, especially in the case of smears and squashes, the results are excellent. With sections, however, phase contrast is not always an efficient substitute for staining. A considerable improvement in accuracy of localization is to be expected from the development of methods in which the position of the radioactive point is established by following the recorded electron track down through the emulsion and on to the section. Emulsions which record electron tracks have recently become available and their application to autoradiography is a matter of technical detail. Many technical improvements have been reported in the literature since the first edition of this book was published. Most of these are included in the valuable treatise by Fitzgerald (1955). Other papers of general technical interest are those of Magnusson and Odeblad (1954), Arnold (1954), Arnold and Webster (1954), Sherman and Essman (1954), Edwards (1955), Kaminski (1955), Siess and Seybold (1957) and Simmel (1957).

Quantitative Autoradiography

This process is based on the fact that in track emulsions each particle hitting the film will produce one track. If the number of particles produced per decaying atom of the radioactive isotope is known then the content of radioactive material in the tissues can be estimated. Cormack (1955) gives figures for the grain yields in Kodak Stripping Film. The number of grains per unit area is proportional

to the exposure up to approximately 100 grains per 100 μ^2 (Dudley, 1954), if fading of the latent image can be neglected. With longer exposures (3 weeks and more) this is the case.

There are four types of procedure by which the amount of radioactivity in cells and tissues can be calculated :

(1) Grain Counting (Andresen *et al.*, 1952).
(2) Track Counting.
(3) Counting of Labelled Units (e.g. Nuclei).
(4) Photometric Densitometry.

These methods have all been very fully considered by Pelc (1957) and by Levi (1957). The first two, and especially the first, are extremely laborious and the third has only limited applications. It is therefore much preferable to employ the fourth type of method.

An automatic grain counter based on the principle of the flying-spot microscope was designed by Dudley and Pelc (1953) but this apparatus was not suitable for routine use. Levi (1957) has pointed out that direct estimation of the density of grains with a densitometer is impossible, for several reasons. She recommends the use of instruments containing a cylindrical lens, which spreads the grain image into a band (Mazia, Plaut and Ellis, 1955). This method is less open to criticism than most others but still apparently has many shortcomings. Its application to the quantitative histochemistry of histochemical end products deposited in the form of grains, dots or granules (e.g. cobalt-formazans ; Chapter XIX, p. 547) might well be considered.

An alternative instrument, designed by Gullberg (1957) uses a direct recording microscope to produce a photometric trace of the density of the grains in an autoradiograph.

Conclusions

In many fields it is to be expected that the technique of autoradiography will displace the ordinary methods of histochemistry. Localization of antigens (protein) by means of radioactive specific antibody is a field in particular in which great advances are certain to be made. If a pure enzyme is available, for instance, localization of the same enzyme in the tissues can be made by means of radioactive antibody. A high degree of precision is to be expected. This does not mean that the enzyme techniques recorded in Chapters XIII to XXI are likely to be superseded, even in the foreseeable future, for routine applied histochemistry. It may be possible, however, to check by an autoradiographic technique the accuracy of localization which they afford. The ease with which foreign materials, organic or inorganic, which have been introduced into the body, can be localized by autoradio-

graphy suggests that the often very insensitive techniques of conventional histochemistry will be entirely displaced. Specific compounds of the type now used for localizing various reactive groups in the tissues, instead of possessing a coloured or chromogenic group as they do now, will be labelled with a radioactive group or element. A great increase in sensitivity should result in many cases, especially where the colour developed by the usual reaction is poor.

These few examples may serve to indicate that the application of autoradiography to the problems of histochemistry is likely to result in advances at least as great as those which can be expected in most of the other branches of the subject. The routine application of autoradiography, however, will necessarily for many years lag behind its application to research.

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CHAPTER XXVIII

OTHER PHYSICAL METHODS

The physical methods, particularly those concerned with measurement, are of increasing importance in biology and applied histochemistry. With the exception of polarization, phase, and interference microscopy, however, their present use is restricted to a few specialized laboratories. This is partly due to the complexity and expense of the necessary apparatus but also to the fact that in their application many of the instruments are limited to very narrow fields. A general laboratory cannot therefore easily justify the capital expenditure which is required for their development or purchase.

The physical techniques are thus not of transcendent interest to the majority of histochemists but all who practice histochemistry should consider, at all times, whether the application of one or other of these techniques might not add considerably to the value of work carried out with analytical and functional methods of a more chemical nature.

It is fortunate that many texts are available which describe all aspects of the theory and application of physical techniques to biological problems. They deal particularly thoroughly with *Interference Microscopy* and *Absorption Spectrophotometry*. The techniques of *Fluorescence Microscopy* and *Autoradiography* have already been considered in Chapters XXVI and XXVII and in this chapter I propose, therefore, to refer only briefly to the remaining physical techniques which are applicable to histochemical studies. I shall endeavour to provide some indication of the types of problem to which each is suited rather than to deal with them exhaustively. In each case the major references dealing with the techniques are given.

Polarization Microscopy

Several excellent articles on this subject are available. The reader is referred to the chapter by Bennett in McClung's *Handbook of Microscopical Technique*, 3rd Edition 1950, and also to the chapter by Schmidt (1957a) in the *Handbuch der Mikroskopie in der Technik*. Other texts dealing with the theory and application of the polarizing microscope are those of Oster (1955), Barer (1955), and Schmidt (1957b).

For routine purposes an expensive polarizing microscope is unnecessary and its facilities can be provided by two screens of polarized

material, one cut to fit the substage condenser ring, the other fitted into a cap designed to cover the ocular of a monocular microscope. Alternatively, if binocular optics are employed, the second screen is designed to be placed over the slide carrying the section which is being examined. There has recently been considerable interest in the practical problems of dichroism in stained tissues. A simple example of this is the pink colour produced when Congo red-stained amyloid is examined by polarized light (Chapter X, p. 284). The problems of dichroism in tissues stained with a variety of dyes (Evans blue, Amidoschwarz 10 B, Congo red, Chlorantin fast green BL, Methyl orange) have been considered by Diezel and Neimanis (1959). These same authors (1957a and b) described metachromatic staining effects in lipids after staining with Sudan black B.

According to Schmidt (1932, 1940), dichroism is a *sensitive index of orientation* in tissues and, in some cases, it may reveal a regular array of the molecules which escapes polarization studies.

Phase and Interference Microscopy

This subject has produced a very large volume of recent literature. Chapters concerning it have been written in *Analytical Cytology* (Barer, 1955) and in *Physical Techniques in Biological Research* (Osterberg, 1955). A text which is particularly acceptable to biologists, and which is strongly recommended, is that of Hale (1958). This author discusses and explains the merits and demerits of the various types of interference microscopes which are commercially available and deals particularly thoroughly with the practical applications of interference microscopy, including its quantitative aspects.

In the proceedings of a symposium on *Cytochemical Methods with Quantitative Aims* there are a number of critical papers discussing many aspects of interferometry and refractometry (Barer and Dick, 1957 ; Davies, Deeley and Denby, 1957 ; Ingelstam, 1957 ; Johansson, 1957 ; Svensson, 1957). In conjunction with Hale's book, these papers present the reader with a more or less complete survey of the modern applications of interference microscopy.

The main value of interference microscopy is that it permits measurements to be made of the total dry mass of an object. This can be estimated to within $0{\cdot}5 \times 10^{-12}$ g. by interferometry. The only alternative method is by X-ray absorption (Historadiography) as described by Engström (1946, 1955) and Clemmons (1957). The measurement of dry weight is important in histochemistry as a basis for the quantitative expression of results obtained with a variety of techniques.

Interference microscopy also allows the measurement of mass per unit area and of the refractive index of the material of which an

FIG. 238. Hallén Mikrokator for thickness measurements on thin tissue sections.

[*To face p.* 757.

object is composed. From this the concentration of material in the object can be calculated. Finally, it provides a method for calculating the thickness of an object, and hence its volume. It should be possible to measure thickness to within ± 150 Å. As pointed out by Hale (1958) interference microscopy can be used as an isolated technique but it is much more valuable when used in conjunction with other techniques, such as microspectrophotometry.

The refractive indices of tissue components were calculated by Hancox and Kruszynski (1956), using phase contrast microscopy and fluids of known refractive index. This type of study should prove useful in applied histochemistry but it is technically difficult to provide and maintain a series of fluids with stable refractive indices.

Thickness Measurements

There are four ways in which the thickness of microscopic objects can be measured (Lange and Engström, 1954).

(1) Focussing on lower and upper surfaces.
(2) Stereomicroradiography.
(3) Mechanical methods.
(4) Interferometry.

The first of these can be carried out with the calibrated fine adjustment of an ordinary microscope. The error entailed, however, is at least 10 per cent. for sections 5 μ thick, according to Lange and Engström (1954). On the other hand Brattgård (1954) calculated the maximum error in 5 μ sections as 3·2 per cent. The method seems not to be acceptable, even if the last figure truly represents the accuracy obtained.

Stereomicroradiography requires special apparatus and gives excellent results with thick sections (50–200 μ) but with thin sections it is quite inaccurate. Glimstedt and Hakansson (1951) introduced a mechanical method for measuring section thickness. This depended on the use of a commercially available instrument, the mikrokator, adapted by the provision of a special probe and used with the minimum possible pressure (200 mg.). Even then it is probable that the pressure of the probe on the sections will cause some deformity since 200 mg. on an area 100 × 100 μ corresponds to a pressure of 2 kg./cm.^{2} (Eränkö, 1955). Hallén (1956) modified the mikrokator by substituting a profile microscope for the probe. His instrument* (Fig. 238) is based on the following principle. If a flat surface is focussed in a profile microscope the wire in the plane of the field diaphragm gives a sharp shadow line in the field of view. Alteration of focus up

* Aktiebolaget and Johansson ; Eskilstuna, Sweden.

or down causes lateral displacement of this line which becomes simultaneously weaker in contrast. The lateral movement is symmetrical around its centre position with regard to motion, sharpness and contrast. A cross line in the eyepiece of the microscope indicates the position of the axis of symmetry in the field of view and under these conditions great accuracy can be achieved in focussing on different levels of an object. Under ideal measuring conditions an accuracy of 0·12 μ is possible. Hallén's instrument thus represents the best that can be achieved by the use of profile microscopy.

Interferometric measurement of thickness is highly accurate. A simple method was described by Richards (1947) based on the illumination of coverslip and slide with monochromatic light from above. Interference fringes are seen and the thickness of the section is obtained by multiplying the number of fringes by a factor (0·29 for sodium light). Both coverslip and slide must be optically flat. The figure obtained is an average for the whole section and the error for a single cell may be high. Mellors *et al.* (1953) used a method based on the use of multiple-beam interference fringes to measure the maximum thickness of cells and Barer and Dick (1957) used the combined methods of interferometry (dry mass per unit area) and immersion refractometry (concentration of solids) to measure the thickness of different regions of the cytoplasm in chick fibroblasts. Their paper should be consulted for details.

Relative Volume Measurements

These are considered at length by Eränkö (1955) in his book on quantitative measurements. He describes the use of the planimeter in making area measurements and also the methods of *Line Sampling* (Uotila and Kannas, 1952) and *Point Sampling* (Chalkley, 1943).

Absorption Spectrophotometry (Cytophotometry)

The principles concerned with absorption measurements are set forth in a simple manner by Eränkö (1955) and more fully by Lothian (1949) and Mellon (1950) in two works devoted to the subject. Other papers which describe either visible or ultra-violet spectrophotometry are those of Pollister and Ornstein (1955), Hiskey (1955), Nurnberger (1955) and Scott (1955).

Quantitative spectrophotometry is made possible by the fact that the proportion of light absorbed by a solution is dependent on the concentration of the absorbing substance. The magnitude of the absorption thus gives the concentration of the substance. In chemical analysis the solutions measured are usually clear and the substance is evenly distributed in the solvent employed. Problems of light

refraction and uneven distribution of the absorbing substance therefor do not present themselves.

The proportion of light absorbed by a layer of absorbing material is not influenced by the intensity of incident radiation (Lambert's law) and the magnitude of absorption is, as stated above, a function of the number of absorbing molecules, when these are dissolved in a non-absorbent solvent (Beer's law). If monochromatic radiation is used Lambert's law is valid and, with this proviso, Beer's law is also valid for many of the dyes used in histology and histochemistry. Deviations from it are frequent, however. That is to say many coloured substances absorb less light at high concentrations than they would if they obeyed the law. As Eränkö points out, Beer's law should never be expected to be valid in the case of stained sections.

Errors due to refractive index differences cause a loss of intensity of transmitted light which is not due to the absorbing substance. These have been discussed by Caspersson (1950) and by Glick, Engström and Malmström (1951). Errors due to scattering have been considered by Barer (1952), and those due to the orientation of molecules by Commoner (1949) and by Commoner and Lipkin (1949)

In the simplest form of cytophotometer an image of the cell or part of the cell to be studied is allowed to fall on a photoelectric cell which records the current passing through a galvanometer. The deflection through the object is compared with the deflection produced when the incident light passes through a portion of the section unoccupied by any structure. Simple instruments of this type, such as the apparatus designed by Lison (1950, 1953) and used by Pasteels and Lison (1953) have been extensively criticized by Naora (1951, 1952). The latter pointed out that if a larger area of the object is illuminated than the area actually used for absorption measurements a transmission of stray light may occur due to internal reflections. This is known as the Schwarzchild-Viliger effect. Naora (1955) proposed that this effect should be eliminated by the use of an inverted microscope in place of the substage condenser. Ornstein and Pollister (1952), however, consider that condenser apertures are usually sufficiently small and the results obtained by substituting an inverted microscope (Thompson and Frazer, 1954) did not differ significantly from the original figures. In any case the Lison apparatus is used in many laboratories to obtain relative measurements of stained objects and much valuable work has been done with it.

The formula given by Lison (1950) for the conversion of galvanometer readings into DNA units (Feulgen staining) was found by Wolff (1957) to be inexact. This author prepared an absorption curve for different concentrations of fuchsin and used this to obtain the converted value for DNA. The value for relative DNA content per nucleus is

obtained by multiplying this figure by the area of the nucleus determined with a planimeter.

The most important error in cytophotometry is the distributional error which is due to the fact that the stained substance in the tissues or the product of the enzyme reaction is never homogeneously distributed. This error can be eliminated in three main ways.

Scanning Procedures. In these the absorption is measured from a large number of small points whose size is comparable to the size of the smallest inhomogeneity resolvable by the optical system employed. Thousands of transmission figures are obtained from a single object (i.e. a cell, nucleus or nucleolus) and these must be transferred and integrated in order to reach the overall mean density of staining of the object. This procedure is performed by some electronic computing device.

The simplest integrating microphotometer available is the one designed by Deeley (1955)* but many others have been designed by Caspersson and his group in Stockholm (see Caspersson *et al.*, 1951, 1957). The principle has been used extensively (see Walker, 1953, 1955 ; Hallén and Hydén, 1957 ; Svensson, 1957, and Hutchison *et al.*, 1958). It is customary, in working with scanning microphotometers, to use a crushing condenser of the type described by Davies, Wilkins and Boddy (1954). Compression by means of this device increases the homogeneous area of the object and provides an equal thickness of the object over its whole extent. It must be remembered that accurate photometric measurements are not possible if the density of the object, or of a part of it, exceeds 1·0 (i.e. 10 per cent. of the incident light is transmitted).

Two Wavelength Methods. With these methods the absorption of the object is measured in light of two different wavelengths chosen so that, for the object concerned, they give an absorbance ratio of 1 : 2. Any change in this ratio in actual measurements will be a measure of the distributional error. A means for correcting the latter is thus provided. The development of a two wavelength microscope has been described by Mendelsohn (1958) but the apparatus is not commercially available. An ordinary research microscope can, however, be converted into a two wavelength microscope without much difficulty or expense, except that the provision of a monochromator is essential. Difficulties arise in finding a suitable pair of wavelengths for any particular stain or reaction and Mendelsohn has described a photographic extension of the method to overcome this difficulty in which a photomicrograph is taken at a single wavelength and during development a colour is coupled into the film. The coupling dye is selected so that wavelengths giving 1 : 2 ratios

* Available from Barr and Stroud Ltd., Glasgow W.3, Scotland.

are easily obtained from its absorption curve. A coloured transparency is then placed on the stage of the two wavelength microscope, in place of the slide containing the object, and measurements are carried out as in the original method.

So far, this principle has been used only for determination of the Feulgen and Sakaguchi reactions and for measurements of gallocyanin-stained nuclei. It should, however, be applicable to a wide variety of reactions although complicated calculations are necessary to obtain the true biological values. These, however, are facilitated by a set of tables given by Mendelsohn.

Recently a comparison has been made by Mendelsohn and Richards (1958) of the performance of the Deeley integrating microphotometer and of the two wavelength microscope in measuring the absorbance of a single object (gallocyanin-stained ascites tumour cells). The results indicated that measurements were directly comparable and confidence in both was therefore considerably increased.

Photographic Methods. These depend on the fact that lack of homogeneity in a specimen is truly registered in a photomicrograph. Two different techniques have been employed for making measurements of the photographic image. The first, and simplest, is by scanning the object with a microdensitometer. This technique was used extensively by Kurnick (1950), and also by Frazer and Davidson (1953) and Thompson and Frazer (1954). The double beam instrument described by Walker (1953, 1955) is admirably suited for measuring the density of the image in photographic emulsions since this density is balanced against a density wedge and traces are recorded across the wedge. The optical density of any part of the specimen can then be read from the curve. Provided that all points in the specimen lie on the linear part of the gamma curve for the emulsion the integrated photographic density above the background of the image may be converted into the integrated absorbance factor derived from the gamma curve.

The second photographic method for avoiding the distributional error is by silver analysis of photomicrographs. This principle was originally suggested by Ornstein (1952). The following procedure is carried out. Test plates are first exposed through a clear region of the slide on which the object is mounted. The emulsion and the development time are chosen so that the gamma value of the image $= 1{\cdot}0$. The exposure which gives the highest optical density on the linear part of the characteristic curve is chosen as the correct exposure for the negative. A small part of the plate is then masked and the chosen field is photographed. The negative is then developed under the chosen conditions and subsequently the masked area is exposed in an enlarger on to the same type of emulsion, and the exposure

giving the maximum density of the straight line portion of the curve is determined. Using this exposure the whole negative is then printed and developed under the chosen conditions. The image of the structure being studied is then cut out of the print and its silver content is determined chemically. The quantity of silver is proportional to the amount of absorbing material in the structure.

Ornstein's technique was employed by Niemi (1957, 1958) for the measurement of hæmoglobin in red blood cells. This author made several modifications of the original principle which are fully described in his second paper. He found that although the reduced silver was easily dissolved out of the photographic prints difficulties occurred in estimating the amount in solution. The opposite condition is found in the case of dyes (stains or products of histochemical enzyme reactions) deposited in cells. These are often very difficult to elute (especially substantive products of enzyme reactions ; Chapter XIII) and tryptic digestion may be necessary. Once eluted, however, the amount of dye in solution is usually easily determined.

Applied Cytophotometry

It is impossible to cover in a limited space all the applications of spectrophotometry applied to cells and tissues. Some idea can be given, however, of the main emphasis of most of the work recorded in the literature.

Nucleic Acids. Numerous quantitative studies have been made of the Feulgen reaction for DNA (Chapter VIII, p. 193) and this reaction has probably been more studied than any other. This is because the Feulgen reaction has been found to fulfil the essential requirements for cytophotometry (Schrader and Leuchtenberger, 1950 ; Swift, 1950a and b ; Sibatani and Naora, 1953 ; Sibatani, 1954). An excellent review of quantitative Feulgen spectrophotometry is given by Vendrely and Vendrely (1956). In place of the Feulgen reaction methyl green staining has also been used (Pollister and Leuchtenberger, 1949 ; Kurnick, 1950) but this has been much less popular. The dyes Azure A and Azure B have also been used (Flax and Pollister, 1949 ; Flax and Himes, 1950, 1952) and more recently gallocyanin-chromalum (see Chapter VIII, p. 210), originally used for the purpose by Lagerstedt (1948), has become much more popular. In the hands of Sandritter (1952), Oram (1955) and Weber (1958) the reaction has been reported to be accurate and reliable.

Proteins. Proteins other than nucleoproteins have been subjected to quantitative spectrophotometry after staining with Fast green FCF (Schrader and Leuchtenberger, 1950), by Millon's reaction (Pollister and Ris, 1947) and by means of Naphthol yellow (Deitch,

1952). It has been reported by Cafruny *et al.* (1955) that the DDD reaction for SH groups (Chapter V, p. 116) satisfies the major requirements for quantitative histochemistry.

Enzymes. The application of cytophotometric measurements to the determination of enzyme kinctics in single cells is extremely desirable. Histochemical methods for enzymes now exist which fulfil all the necessary kinetic requirements (see Chapter XIII) and yet the conventional method of grading by using a series of arbitrary distinctions (1 +, 2 +, 3 +, 4 +, etc.) is still widely employed. The advantages which would be obtained by the use of cytophotometry in enzyme histochemistry should be sufficiently obvious. In addition to the provision of accurate relative measurements of the enzymic activity of single cells in different states of function it will be possible to investigate the mechanism of enzyme action *in situ* and to calculate the Michaelis-Menten constant of a given enzyme within the cell.

These desirable objectives are frustrated by a number of factors, the most important being the nature of the final reaction product. This is often particulate, sometimes grossly so, and the amount of final reaction product deposited in the cell does not always bear a linear relationship to enzyme activity. Several possibilities exist for overcoming these difficulties. First, it may be possible to design reactions having suitable kinetics and producing a non-particulate product which can be measured by cytophotometric apparatus. (Two wavelength microscope, integrating densitometer.) Secondly, the photographic method of Niemi (1958) may be adapted to the measurement of enzyme reaction products in isolated cells (ascites tumour, macrophage culturcs, etc.). Thirdly, cylinder lens grain counting techniques (Levi, 1957) designed for quantitative autoradiography may be adapted for the measurement of particulate enzyme products such as those which result from the application of the cobalt-formazan techniques for dehydrogenases and diaphorases (Chapters XIX and XX).

It is absolutely essential for the further progress of enzyme histochemistry that the difficulties connected with quantitation by cytophotometric methods be overcome. At present, however, it is impossible to recommend any single method, or any single photometric apparatus, as widely suitable for measurements in cytophotometry. It is no accident that the users of cytophotometric apparatus confine themselves so extensively to measurements of Feulgen-stained nuclei. In many cascs the apparatus which they use is to all intents and purposes restricted to such usage. Those who do not wish to concern themselves with the measurement of DNA in individual nuclei will be well advised to remain, for the moment, on the touchlines of cytophotometry.

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CHAPTER XXIX

ELECTRON HISTOCHEMISTRY

Just as histology becomes histochemistry when chemical interpretations of its reactions become available, so electron microscopy becomes electron histochemistry. Histology, as a Science, has been in existence for over 100 years but electron microscopy is hardly 20 years old. We cannot therefore expect electron histochemistry to have yet developed very far along the road which it will ultimately take.

This chapter is a brief one and it justifies its existence only if it succeeds in drawing attention to the future applications of the electron microscope to histochemical techniques presently available, and to the possibilities for designing new histochemical techniques especially for the electron microscope. The development of new techniques for this purpose has been discussed by Seligman (1956) who frankly stated in his paper that his speculation might well be considered premature. Nevertheless, as he also pointed out, it is necessary to make a start, and his justification for his paper will do equally well for this chapter.

Before dealing with the actual application of the electron microscope to histochemistry it may be well to describe some of the factors with which we must necessarily be concerned. These are connected with the production of contrast in the electron microscope image (Valentine, 1958). The principles of electron microscopy have been adequately presented by Selby (1955), and at somewhat greater length by Cosslett (1955).

Contrast in the Electron Microscope Image

The detail observable under the high magnification of the electron microscope is limited more by lack of contrast than by any lack of resolution in the microscope. The latter can now resolve objects as small as 15 Å, that is to say having a diameter of only a few atoms. Various techniques are used for increasing contrast in specimens by artificial means and these include treatment with various reagents and metal shadowing of the surface. The latter cannot be employed in electron histochemistry but the former, by analogy with histological practice, has come to be known as "electron staining". This term, though it is not a very accurate one, is convenient and it will probably continue to be used for this reason.

The precise physical property which makes any compound useful

as an electron stain is still far from clear. Some authorities have gone so far as to claim that all substances are almost equally efficient in increasing contrast by increasing electron density. The problem is made more difficult by the fact that exact measurements of contrast in the electron image are not easy to make (Hall, 1958) and, except in rare cases of very intense staining, subjective opinions are obviously unreliable.

A simple theoretical approach to the problem of contrast has been made by Zeitler and Bahr (1957) and from their formulæ Valentine (1958) has deduced the conditions necessary to produce a just appreciable increase in contrast in the image of any part of a specimen.

If the object under consideration covers an area of S cm.^{2} at right angles to the beam then we may deduce that an increase in contrast requires the addition of N atoms of atomic number Z uniformly over or through the object where $NZ^{4/3}/S = 5{\cdot}5 \times 10^{17}$ for a microscope working at 60 kV ($4{\cdot}7 \times 10^{17}$ for 50 kV ; $8{\cdot}25 \times 10^{17}$ for 100 kV). By rewriting this formula in simple terms one can predict the effect of adding various amounts of different substances to the specimen and one can, to some extent, reconcile conflicting views on requirements for increasing contrast. There are three useful bases on which contrast can be compared and it is important to distinguish which is relevant in any particular case.

Contrast on the Basis of Equal Thickness. This is relevant particularly when selecting a material for shadowing a specimen and for calculating the minimum thickness of deposit which is required. It has little direct concern with electron histochemistry, but the calculations made by Valentine led him to suggest that a useful histochemical technique for the electron microscope would be one which formed small deposits of some dense substance.

Contrast on the Basis of Equal Weight. If an object d cm. thick of density ρ g./cm.$^{-3}$ can bind p per cent. by weight of an added substance, then a detectable increase of contrast requires p to have a value not less than that given by $(p/100)\rho d\mathrm{L}Z^{4/3}/\mathrm{A} = 5{\cdot}5 \times 10^{17}$ where A is the atomic weight of the added substance and L is Avogadro's number ($6{\cdot}02 \times 10^{23}$). Taking $Z^{4/3}/\mathrm{A}$ and the density of the specimen as unity, $p = 9 \times 10^{-5}/d$. Thus for a specimen of given thickness the percentage of an electron stain required to increase contrast appears to be independent of the nature of the stain used. For an object $0{\cdot}5$ μ thick such as a bacterium a 2 per cent. uptake of any stain should theoretically increase the contrast sufficiently and Valentine suggested that the usual histological stains should be tested by means of the electron microscope. For a thin (200 Å) section, on the other hand, the above calculations indicate that the addition of 45 per cent. of stain is required to increase contrast.

Calculations of this type were made by Ornstein (1957) who deduced that, since osmium is only taken up to the extent of some 10 per cent. by tissues fixed in osmium tetroxide, "staining" of objects in thin sections by this means must be fictitious. On the basis of practical observations most electron microscopists would quarrel with this conclusion.

Contrast on the Basis of Equal Numbers of Molecules. If the object to be stained is d cm. thick and can bind n molecules of stain per cm.^{3} then to obtain an increase of contrast it can be calculated that $nd(\Sigma Z^{4/3}) = 5{\cdot}5 \times 10^{17}$ where $(\Sigma Z^{4/3})$ is the effective value of $Z^{4/3}$ obtained by adding up the effective value of $Z^{4/3}$ for each atom in the molecule of the stain. If, as is often the case, the molecules of the stain are bound to the surface of membrane and not uniformly distributed, as has been assumed above, then if n molecules are bound per cm.^{2} of membrane surface, the normal to which makes an angle θ with the beam at any point, contrast will be obtained when $n(\Sigma Z^{4/3})\sec.\ \theta = 5 \times 10^{17}$. When tissues are fixed with OsO_4 the latter is bound as OsO_2 ($\Sigma Z^{4/3} = 353$) and if $\theta = 89°$, as where the membrane concerned runs at right angles to the surface, then $n = 2{\cdot}5 \times 10^{13}$cm.$^{-2}$. This means that one osmium atom per 400 Å^2 of surface will increase its contrast. This figure is much more reasonable than those calculated in the preceding section and osmium staining of surface membranes may therefore be a fact.

The foregoing considerations may be summarized by saying that when substances are added to a specimen in layers of equal thickness the increase in contrast will be proportional to the density of each substance. If, however, equal weights of substances are added, the changes in contrast will be almost the same in each case. If equal numbers of molecules are added the increase in contrast will depend on the sum of $Z^{4/3}$ for each atom in the molecule, that is to say on the molecular weight.

Application of Electron Microscopy to Existing Techniques

Most of the applications of electron microscopy to existing techniques (involving no change in principle) have been to enzyme techniques having metallic salt precipitates as their product. Gomori's (1941) technique for acid phosphatase (Chapter XV, p. 433) was performed on tissues, fixed briefly in 1 per cent. OsO_4, by Sheldon, Zetterquist and Brandes (1955). A similar technique for alkaline phosphatase, using the von Kóssa procedure as the final step, was carried out by Brandes, Zetterquist and Sheldon (1956). Both these methods resulted in the appearance in the E.M. of a dense granular precipitate which, in the case of alkaline phosphatase, was seen to line the spaces between the microtubules composing the brush

FIG. 239. Rat heart muscle. Incubated in standard dehydrogenase medium containing Nitro-BT. Fixed in buffered osmium tetroxide and embedded in methacrylate. Intramitochondrial formazan deposits. Electron photomicrograph. × 30,000.

FIG. 240. *E. coli*. Incubated for two seconds in standard MTT-cobalt medium containing succinate. Formalin-fixed. Electron photomicrograph. × 22,000.

FIG. 241. *E. coli*. Incubated for 30 seconds in standard MTT-cobalt medium containing succinate. Formalin-fixed. Light photomicrograph. × 81,00.

[*To face p.* 768.

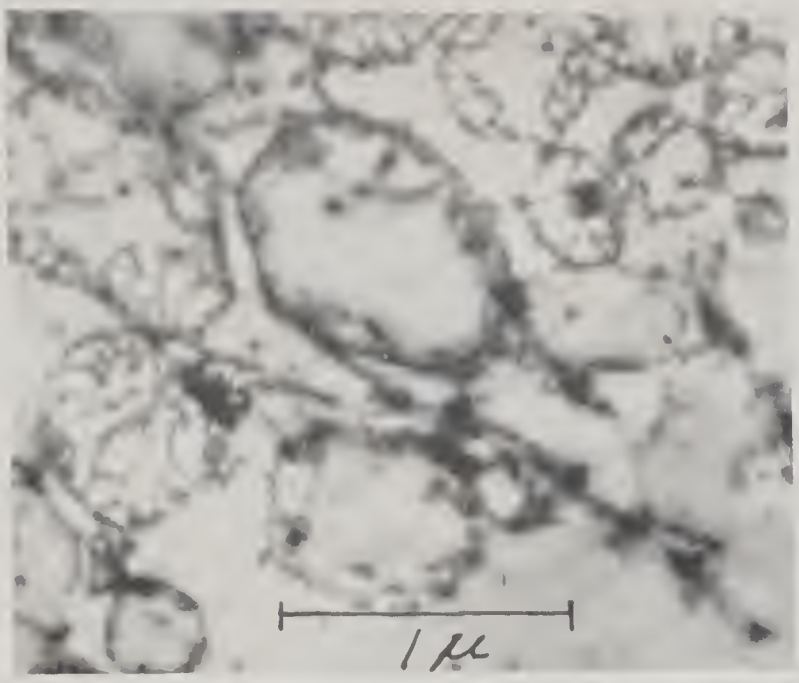

FIG. 242. Rat kidney. Incubated for 5 minutes in standard medium containing DPNH, MTT, and cobalt. Fixed in buffered osmium tetroxide. Subsequently treated to convert cobalt-formazan to CoS. Electron dense deposits appear at intervals along the mitochondrial membranes. Electron photomicrograph. × 28,000.

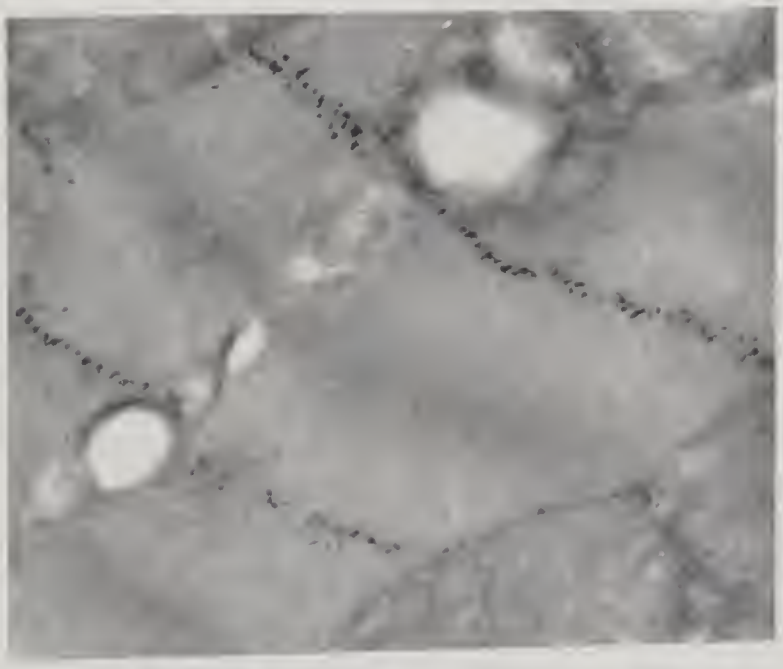

FIG. 243. Rat striated muscle. Shows the granular deposits which indicate the "M-band Enzyme". Electron photomicrograph. × 30,000.

border of mammalian intestinal epithelium. Studies of the same type were carried out on the ATP-ase (Chapter XIV, p. 418) by Nelson (1958). The localization of this enzyme in rat spermatozoa was demonstrated.

The electron histochemistry of dehydrogenases has been investigated by three different methods with results which have hitherto left a great deal to be desired. Barrnett and Palade (1957) used potassium tellurite, which is reduced with some difficulty by tissue dehydrogenase systems. They were nevertheless able to show the final product in thin electron microscope sections as fine needles (crystals) and small dense particles. In both cases these were closely associated with the mitochondrial cristæ.

A second method has been to study the increase of contrast obtained by the deposition of the formazans of nitro-BT or nitro-NT (Chapter XIX, p. 545) in the mitochondria. Because of its insolubility in organic solvents and in methacrylate these products can be carried through the conventional embedding processes for electron microscopy. The results of such a study have been reported by Sedar and Rosa (1958), and also by Nelson (1959), and by Barrnett (1959). The results obtained by the last of these authors are shown in Fig. 239, p. 768, where the deposits of dinitroformazan can be observed to be apparently randomly distributed within the mitochondrion.

Attempts have also been made to demonstrate the presence in mitochondria of the cobalt formazan of MTT (Chapter XIX, p. 547). In the early stages of development these were frustrated by the solubility of the compound in organic solvents. The increase of contrast due to deposition of cobalt formazan in bacteria could, however, be demonstrated without embedding and sectioning. This was not found possible with isolated mitochondria since their intrinsic electron density was too great. The result of incubating *E. coli* in the standard succinic dehydrogenase medium for 2 seconds is shown in Fig. 240, p. 768. After 30 seconds' incubation the product was visible under the light microscope (Fig. 241). This demonstration makes it clear that when the product of any enzyme reaction is visible by light microscopy the result represents a gross over-incubation. The electron microscope thus offers an escape from the problems of false fine localization due to over-incubation but it carries with it a number of new problems. These are connected with diffusion of the product during processing and with the fact that non-specifically adsorbed substrate, product or capture reagent may now give sufficient contrast to be visible. Distinction between true and false increase of contrast may then be a matter of some difficulty.

More recently it has proved possible to preserve the cobalt-formazan deposits from MTT in the mitochondria. This has been

achieved by fixing small blocks of tissue after incubation, in osmium tetroxide. Following fixation the cobalt is converted into its sulphide by brief treatment with yellow ammonium sulphide before proceeding to dehydration and methacrylate embedding. The result (Pearse and Scarpelli, 1959) is illustrated in Fig. 242, p. 769. It shows that the deposition of cobalt formazan is largely upon the internal surfaces of the mitochondrion (i.e. the cristæ mitochondriales).

Many other enzyme reactions (e.g. the indoxyl esterases) should prove applicable to electron microscopic study. Such studies are urgently required, especially in the dehydrogenase field, where it is necessary to confirm or deny the intramitochondrial localization of dehydrogenases and of cytochrome oxidase. Modern techniques using the light microscope, which are discussed in Chapters XVIII, XV and XX, indicate that in both cases enzymically active areas are separated from each other by areas free from activity (Pearse and Scarpelli, 1958).

A presumed acetylcholinesterase has been shown by Barrnett and Palade (1958) using the thiolacetic acid technique (Chapter XVI, p. 480). Their electron photomicrographs showed the enzyme not only in the motor end plates and mitochondria of rat muscle but also in the form of discrete deposits in the M-band of each myofibril in each sarcomere. These very striking appearances are shown in Fig. 243, p. 769.

Application of New Techniques in Electron Histochemistry

The problem of OsO_4 as an electron stain has been considered very fully by Bahr (1954, 1955) and also by Houck and Dempsey (1954). Since OsO_4 is the standard fixative for electron microscopy the results of its use are not usually subjected to histochemical analysis, however. Phosphotungstic acid has been employed extensively as an " electron stain ", especially for muscle (Schmitt *et al.*, 1945 ; Hodge, 1956) but once again histochemical interpretation has not usually been made. Chromium compounds have been studied by Low and Freeman (1956) and they are recommended for nucleic acid studies. Ferric salts were used for the same purpose by Bernstein (1956). The localization of silver was studied by Dempsey and Wislocki (1955) after administration of dilute $AgNO_3$ to rats. Dense deposits were found in the basement membranes and also in fixed and free macrophages.

Potassium permanganate was first described as a fixative for electron microscopy by Luft (1956) and it was used by Usuku (1958) in studies of elastic fibres. In all these cases, although " electron staining " was achieved no chemical explanations were offered.

Modifications of existing methods of analytical histochemistry designed to show specific groups in tissue proteins have been described

by several authors. Bradfield (1954) used a modified Feulgen reaction (Chapter VIII, p. 193) in which the aldehydes resulting from acid hydrolysis were demonstrated by means of an ammoniacal silver hydroxide. With this method the localization of DNA in bacteria was investigated. A method for 1 : 2-glycol groups has been described by Dettmer and Schwarz (1953) and Bahr and Moberger (1954) used methyl mercury iodide as a specific " electron stain " for SH groups in striated muscle and plantar epidermis. Tests made with egg albumin *in vitro* suggested that the label might be used with confidence.

Designing of New Techniques for Electron Histochemistry

Many workers feel that the application of electron microscopy to the end products of enzyme reactions may possibly merely magnify the artifact seen with the light microscope. Attention has therefore been directed to the designing of new methods, especially for protein end-groups, which will not be subject to this criticism. Lamb *et al.* (1953) developed a technique depending initially on the performance of the DNFB reaction (Chapter V, p. 86) followed by multiple procedures ending in the deposition of silver, or lead. Seligman (1956) suggested a number of possible ways in which protein end-groups could be demonstrated under the electron microscope. He suggested that electron-dense diazonium salts might be used to reveal aromatic amino-acids capable of coupling with these and that the hydroxynaphthaldehyde method for NH_2 groups (Chapter V, p. 88) might also be modified for electron histochemistry by the use of electron-dense coupling agents. His methods for carboxyl groups (p. 89) and for SH groups (p. 118) are suitable for similar treatment.

Seligman further suggested the use of electron-dense couplers for localizing aldehydes produced by the oxidation of carbohydrates (see above) and fixed lipo-proteins, by interposition of the naphthoic acid hydrazide reagent (Chapter XII, p. 338).

As a general principle Seligman advocated the introduction of iodine (atomic number 53) into reagents intended for use as electron histochemical couplers but he also favoured the re-designing of various reactions to give a final product capable of forming chelates with metals, preferably those having a high atomic number.

Electron histochemistry is now in its earliest stages. Future developments can be forseen which will result in the application of the electron microscope to almost every field of orthodox microscopical histochemistry. These cannot fail to be productive. Just as the electron microscope will ultimately be found to be as necessary a tool in routine pathological laboratories as it is now in those devoted to anatomy, so the extension of the instrument to histochemistry and its use in histochemical laboratories can be regarded as inevitable.

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APPENDIX 1

CHAPTER I has no Appendix ; this is a General Appendix and it contains, *inter alia*, technical details of methods of preparation, embedding, mounting, etc., which do not readily fit into the other Appendices. A selection of buffer tables, covering all the buffers in common histochemical use, is also given.

METHODS FOR THE PREPARATION OF TISSUES

Polyethylene Glycol Wax Embedding for the Demonstration of Lipids and Enzymes

Blank (1949) and Blank and McCarthy (1950) described a rapid embedding technique for histological purposes using a melted mixture of 1 part Carbowax 1500 and 9 parts Carbowax 4000 (50°–54°). Other authors (Berlin and Brines, 1951 ; Firminger, 1950) used similar techniques and obtained thin sections (1–3 μ) in which lipids were well preserved. The method was also used for plant tissues by Van Horne and Zopf (1951) and by McLane (1951), the latter reporting preservation of enzymes in unfixed material embedded in frozen aqueous Carbowax 4000. Rinehart and Abu'l-Haj (1951), following other workers in this field, have described a method for dehydration and embedding which will be found useful for studies of lipids. For work on enzymes, where these are sufficiently stable to heat, the polyethylene glycol wax methods promised to be useful also. In practice, however, with alkaline phosphatase at all events, considerable diffusion of enzyme into the embedding medium and into the surrounding tissue structures has been observed to occur. With other enzymes (acid phosphatase, peroxidases) diffusion is much less evident. Details of the method are given below ; modification of the technique by the addition of some of the higher M.P. waxes (M.W. 1540–4000) may be necessary in hotter climates.

Preparation of Solutions

(A) Stock Solutions of Polyethylene Glycol Wax 1000 *

These are prepared by heating the wax until vapour appears on the molten surface. Water is then added at 50° to make a 70 per cent. and a 90 per cent. solution. These solutions, and the molten 100 per cent. wax, should be kept in an oven at 48°–52°.

(B) Solution for Floating Out

Diethylene glycol	40 parts
Distilled water	50 parts
40 per cent. formaldehyde	10 parts

(C) Affixative Medium

Gelatin (granular)	10 g.
Distilled water	60 ml.
Glycerine	50 ml.
Phenol	1 g.

* The whole range of polyethylene glycol waxes is available from the Watford Chemical Co. Ltd., Copperfield Road, London, E.3, and from General Metallurgical and Chemical Ltd., Moorgate, London, E.C.2. (Known as Carbowaxes in the United States, they are there available from the Carbide and Chemical Corporation.)

Dissolve the gelatin in water at 32°–35°, add glycerine, filter through gauze and add phenol. Apply a thin film to the slides and wipe well.

Method

(1) Fix tissues in neutral 6 per cent. formalin (24–48 hours at room temperature for lipids, 16 hours at 4° for many enzymes).

(2) Trim blocks to 3 or 4 mm. thick.

(3) Place blocks in 70 per cent. wax for 30 minutes, with occasional stirring.

(4) Transfer to 90 per cent. wax for 45 minutes, with occasional stirring.

(5) Transfer to 100 per cent. wax for 60 minutes, rotating the block and stirring.

(6) Embed in wax in paper cups, and harden at 4°.

(7) Cool blocks before cutting and use only dry implements.

(8) Cut sections 4–6 μ (or thicker or thinner as desired).

(9) Float out sections on solution B.

(10) Mount on gelatinized slides (C) ; allow to dry at 37° for 10 minutes.

After sectioning the block face should be coated with wax and the block stored in a dry cold atmosphere. Röhlich (1956) has used this method, embedding for 4–5 hours at 42°, for studies of acetylcholinesterase by the Koelle method (Appendix 16, p. 890). He found a relatively rapid fall in the amount of enzyme demonstrable after storage, and after 1 month could scarcely obtain any reaction at all. Miles and Linder (1953), after testing various polyethylene glycol mixtures, developed a method using Nonex 63B (polyethylene glycol 1000 monostearate) at 39° after preliminary infiltration of tissues with a softer wax, polyethylene glycol 900, at 28°. They found excellent preservation of alkaline phosphatase in cold acetone-fixed tissues but noted some diffusion.

Effect of Temperature and Relative Humidity

The chief drawback to the use of water-soluble waxes is the difficulty of cutting satisfactory sections even after careful experiment to determine the optimum proportions of waxes for a particular climate. Hale (1952) investigated this problem and found that, for sections cut at 7 μ, at 25°, ribboning ceased when the relative humidity reached 40 per cent. and that no sections could be cut above 56 per cent. humidity. At 20° ribboning continued up to 55 per cent. humidity. As a result of these experiments Hale recommended cutting in a cool room (below 18°) so as to be independent, as far as possible, of atmospheric humidity.

Gelatine Embedding for Enzyme Histochemistry

This process is usually carried out when it is necessary to support delicate tissues so that sections can be cut on the freezing microtome. When sections of delicate tissues are required for enzyme studies, and particularly for the coupling azo dye techniques (Appendices 14, 15 and 16), a modified gelatine embedding procedure, designed to damage the enzymes as little as possible, can be employed. This is described below.

Method

(1) Fix thin slices or portions of tissue in 15 per cent. cold neutral formalin (4°) for 10–16 hours.

(2) Wash in running water for 30 minutes.

(3) Embed in gelatine * for 1 hour at 37°.

(4) Cool and harden in formalin (40 per cent. formaldehyde) for 1 hour at 17°–22°. Wash.

(5) Store at 4° or below until frozen sections are required.

Provided that the gelatine block surrounding the tissues is more than a few mm. thick, the hardening process does not affect the enzyme. It should not be prolonged, however, beyond the time indicated.

MOUNTING MEDIA

This section is not meant to be comprehensive ; only those media which are mentioned in the text and in the various Appendices are included. For a comprehensive review of the available permanent mounting media the reader is referred to the Interim and Final Reports of the Committee on Mounting Media (Lillie *et al.*, 1950, 1953).

DPX (Kirkpatrick and Lendrum, 1939, 1941)

Distrene 80 (British Resin Products Ltd.)	10 g.
Dibutylphthalate	5 ml.
Xylene	35 ml.

This is a useful mountant for general purposes which does not cause fading with the majority of stains. Shrinkage is considerable and only a thin layer must separate the section from the coverslip. Thicker layers cause distortion of the microscopic image. The original DPX mountant could not be used for preparations containing silver deposits (reticulin, Gomori's acid and alkaline phosphatase methods), or for sections finished by the cobalt sulphide technique (acid and alkaline phosphatase, Gomori's cholinesterase method).

The new DPX, in which tri-orthocresyl phosphate is no longer used as plasticizer, can be employed successfully for the above-mentioned preparations.

Karo Corn Syrup (Corn Products Ltd.) (Patrick, 1936)

Corn syrup	1 part
Distilled water	1 part

Add a crystal of thymol and keep at 4°.

Chrome Glycerin Jelly (Glychrogel) (Zwemer, 1933)

Distilled water	80 ml.
Glycerine	20 ml.
Gelatin (powdered)	3 g.
Chrome alum	0·2 g.

Dissolve the chrome alum in 30 ml. of water and the gelatin in 50 ml. of water. Mix and add the glycerine. Test pH before use and adjust to pH 7·4 with $NaHCO_3$ if necessary.

* Gelatine pulv. 15 g.
Glycerine 15 ml.
Distilled water 70 ml.
Thymol A small crystal

This mountant shrinks and sets very hard. Removal of the coverslip and renewal of the medium is therefore impossible.

Glycerine Jelly (Roulet, 1948, after Kaiser, 1880)

Dissolve 15 g. best quality white gelatine in 100 ml. distilled water, with moderate heating. Add 100 g. glycerine and warm for 5 minutes on a waterbath, filter hot through glass wool. To each 100 ml. of the mixture add 1 drop of phenol (Phenol liquefactum B.P., U.S.P.).

This medium is kept at 37° in an incubator and applied to the wet section, mounted on its slide, with a thick glass rod. (In cold weather it is sometimes an advantage to warm the slide in running hot water before applying the jelly.) A coverslip is then lowered gently over the medium and the excess is wiped away with blotting paper. The refractive index of glycerine jelly is 1·47, and although it sets hard and has no tendency to draw up under the edges of the coverslip, it can easily be removed by dipping the slide in hot water.

Apáthy's Gum Syrup (Lillie and Asburn, 1943)

Dissolve 50 g. gum acacia and 50 g. cane sugar in 100 ml. water with frequent shaking at 55°. Restore original volume with water. Add 15 mg. menthiolate (sodium ethylmercurithiosalicylate) or 100 mg. thymol as a preservative.

Polysorbitol Medium (Karion-Merck)

Karion is a proprietary medium which can be used for mounting frozen sections. It has a tendency to crystallize on prolonged storage even when the coverslips are ringed.

DECALCIFICATION FOR HISTOCHEMICAL PURPOSES

The standard methods of decalcification using nitric, hydrochloric or acetic acids are usually quite unsatisfactory if histochemical methods of enquiry are proposed. This refers not only to enzyme techniques but also to analytical methods for nucleic acids and polysaccharides and, to a lesser extent, to other methods.

With the three standard methods given below adequate fixation is an important prerequisite. Enzymes which survive this are largely if not entirely destroyed, even when the decalcifying process is short, as when only minor calcium deposits have to be removed.

De Castro's Fluid

Chloral hydrate	50 g.
Absolute alcohol	300 ml.
Water	670 ml.
Nitric acid (conc.)	30 ml.

Jenkins' Fluid

Hydrochloric acid (conc.)	80 ml.
Acetic acid	60 ml.
Chloroform	200 ml.
Alcohol, 95 per cent.	1,460 ml.
Water	200 ml.

With the above fluids treat thin well-fixed blocks for 16–48 hours, as necessary, and post-fix for 48 hours in 10 per cent. neutral formalin. Prolonged decalcification interferes with nuclear staining and Gram staining.

Clarke (1953) Method

(1) Fix tissue in 10 per cent. neutral formalin for 48 hours.
(2) Decalcify in a mixture of equal parts of 45 per cent. formic acid and 6·8 per cent. sodium formate (pH 2·0). Change solution daily (7–14 days).
(3) Wash in water.
(4) Dehydrate, clear and embed as usual.

Electrolytic Decalcification (Richman *et al.*, 1947)

With this method a 2–6 volt direct current is passed through the decalcifying solution (1 part 10 per cent. formic acid and 1 part 8 per cent. HCl). The positive electrode must be platinum, the negative electrode can be carbon. If the temperature of the bath exceeds 40° considerable damage occurs, but even at lower temperatures much protein, nucleoprotein and polysaccharide is extracted.

Decalcification takes 2–6 hours for thin slices (1 cm.) of normal bone, but may take up to 18 hours for dense bone specimens. Treatment with neutral or alkaline 10 per cent. formalin should follow decalcification by this method.

Decalcification with Enzyme Preservation (after Lorch, 1946)

This method was designed for the preservation particularly of alkaline phosphatases.

Method

(1) Fix in two changes of absolute acetone at 0°–4° (thin blocks).
(2) Decalcify for 3–10 days in Lorch's fluid :

Citric acid (crystalline) . . .	14·7 g.
0·2 N-NaOH	700 ml.
0·1 N-HCl	300 ml.
1 per cent. zinc sulphate . .	2 ml.
Chloroform	0·1 ml.

(3) Wash in distilled water, several changes.
(4) Reactivate the enzyme by treatment with 0·1 M-veronal-acetate buffer (pH 9) for 6–8 hours.
(5) Dehydrate in alcohol.
(6) Clear in chloroform.
(7) Embed *in vacuo* in 48° wax.

Decalcification with Enzyme Preservation (after Greep *et al.*, 1948)

This method, like that of Lorch, was designed for the preservation of alkaline phosphatases.

Method

(1) Fix thin slices of bone or whole bones from small animals in absolute alcohol at 4° for 24 hours.
(2) Rinse briefly in 50 per cent. alcohol and in distilled water.
(3) Decalcify in a mixture of equal parts of 2 per cent. formic acid and 20 per cent. sodium citrate for 2–10 days. (Change solution every 3–4 days.)

(4) Rinse in three changes of tap water.

(5) Reactivate by immersion in 1 per cent. sodium diethylbarbiturate for 24–48 hours.

(6) Rinse in tap water.*

(7) Dehydrate in alcohol, clear in benzene and embed *in vacuo* in 48° wax.

The preservation of alkaline phosphatase during decalcification has been considered by a number of authors. The most important papers are those of Greep *et al.* (1948), Zorzoli (1948), Lillie (1951), Majno and Rouiller (1951), and Schajowicz and Cabrini (1954). These last authors (1955) also tested a number of decalcifying solutions with respect to their effects on the histochemical staining of bone and cartilage. Among the solutions tested were 10 per cent. HCl and 7·5 per cent. HNO_3 (1 hour, 24 hours, 5 days), Greep's fluid and Lorch's fluid, for 1 hour, 24 hours, 5, 15 and 30 days, and lactic and perchloric acids for varying times. Sections were stained with toluidine blue, PAS, Hale's dialysed iron, aldehyde fuchsin, mucicarmine and muci-hæmatein. Schajowicz and Cabrini found that with the strong acids there was a rapid loss of metachromasia in cartilage matrix, complete by the fifth day. Glycogen was quickly lost but PAS staining hardly modified. Greep's fluid produced similar changes but much more slowly, scarcely any effect being visible until after the fifth day. Lorch's fluid was observed to produce a phenomenon described as "inversion." Within 7 days an intense metachromasia appeared in bone and osteoid, while that of cartilage was abolished. These observations produced the recommendation that for mucopolysaccharide studies thin pieces of bone should be treated for up to 5 days with Greep's fluid.

For the preservation of alkaline phosphatases Schajowicz and Cabrini (1955) obtained excellent results with Greep's fluid followed by reactivation with sodium barbiturate-glycine buffer.

The Use of Chelating Agents

An ion exchange resin was used by Dotti, Paparo and Clarke (1951) for the decalcification of bone, and many other authors have followed this lead in using organic chelators for the same purpose (Hahn and Reygadas, 1951 ; Sereebny and Nikiforuk, 1951 ; Birge and Imhoff, 1952 ; Hilleman and Lee, 1953 ; Freiman, 1954). The chelator universally employed is the disodium salt of ethylenediamine tetra-acetic acid (Versene, Sequestrene).

Hillemann and Lee Method (1953). (Average time for rat femur, 4 weeks)

(1) Fix in neutral formalin for 24 hours at room temperature.

(2) Transfer to 5·5 per cent. versene in 10 per cent. formalin, renewing the solution every 10 days.

(3) Dehydrate in alcohol, clear, embed in paraffin wax.

Freiman (1954) Method. (Suitable for enzyme studies)

(1) Fix thin slices (or whole bones from small animals) in 80 per cent. alcohol at 4° for 24 hours.

(2) Wash in tap water.

(3) Decalcify in 5 per cent. aqueous E.D.T.A.,† adjusted to pH 6·0–6·5 with NaOH, for 24 hours at 4°.

* Frozen sections may be cut at this stage for enzyme demonstrations.

† Ethylenediamine tetra-acetic acid.

(4) Change solution and keep at room temperature for 2–21 days, changing the solution at regular intervals.

(5) Wash in water.

(6) Reactivate enzyme by treatment with 1 per cent. sodium barbiturate for 24 hours.

(7) Dehydrate, clear and embed *in vacuo* in 48° wax.

BUFFERS

pH 0·65 to 5·20

Walpole (1914). 50 ml. N-sodium acetate + x ml. N-HCl, made up to 250 ml.

pH	x ml. HCl	pH	x ml. HCl	pH	x ml. HCl
0·65	100	1·99	52·5	3·79	42·5
0·75	90	2·32	51·0	3·95	40·0
0·91	80	2·64	50·0	4·19	35·0
1·09	70	2·72	49·75	4·39	30·0
1·24	65	3·09	48·5	4·58	25·0
1·42	60	3·29	47·5	4·76	20·0
1·71	55	3·49	46·25	4·92	15·0
1·85	53·5	3·61	45·0	5·20	10·0

pH 2·2 to 8·0

McIlvaine (1921). 200 ml. mixtures of x ml. 0·2 M-Na_2HPO_4 with y ml. 0·1 M-citric acid.

x ml. Na_2HPO_4	y ml. citric acid	pH	x ml. Na_2HPO_4	y ml. citric acid	pH
4·0	196	2·2	107·2	92·8	5·2
12·4	187·6	2·4	111·5	88·6	5·4
21·8	178·2	2·6	116·0	84·0	5·6
31·7	168·3	2·8	120·9	79·1	5·8
41·1	158·9	3·0	126·3	73·7	6·0
49·4	150·6	3·2	132·2	67·8	6·2
57·0	143·0	3·4	138·5	61·5	6·4
64·4	135·6	3·6	145·5	54·5	6·6
71·0	129·0	3·8	154·5	45·5	6·8
77·1	122·9	4·0	164·7	35·3	7·0
82·8	117·2	4·2	173·9	26·1	7·2
88·2	111·8	4·4	181·7	18·3	7·4
93·5	106·5	4·6	187·3	12·7	7·6
98·6	101·4	4·8	191·5	8·5	7·8
103·0	97·0	5·0	194·5	5·5	8·0

pH 3·6 to 5·6

Walpole (1914). 200 ml. mixtures of 0·1 N-acetic acid and 0·1 N-sodium acetate.

pH	0·1 N-acetic acid (ml.)	0·1 N-sodium acetate (ml.)	pH	0·1 N-acetic acid (ml.)	0·1 N-sodium acetate (ml.)
3·6	185	15	4·8	80	120
3·8	176	24	5·0	59	141
4·0	164	36	5·2	42	158
4·2	147	53	5·4	29	171
4·4	126	74	5·6	19	181
4·6	102	98			

pH 5·29 to 8·04

Sörensen (1909–12). 100 ml. mixtures of M/15 Na_2HPO_4 amd M/15 KH_2PO_4.

pH	M/15 Na_2HPO_4 (ml.)	M/15 KH_2PO_4 (ml.)	pH	M/15 Na_2HPO_4 (ml.)	M/15 KH_2PO_4 (ml.)
5·29	2·5	97·5	6·81	50	50
5·59	5	95	6·98	60	40
5·91	10	90	7·17	70	30
6·24	20	80	7·38	80	20
6·47	30	70	7·73	90	10
6·64	40	60	8·04	95	5

pH 7·8 to 10·0

Clark and Lubs (1916). 50 ml. 0·2 M-H_3BO_3 + 0·2 M-KCl + x ml. 0·2 M-NaOH, diluted to 200 ml.

pH	x ml. 0·2 M-NaOH	pH	x ml. 0·2 M-NaOH
7·8	2·65	9·0	21·40
8·0	4·00	9·2	26·70
8·2	5·90	9·4	32·00
8·4	8·55	9·6	36·85
8·6	12·0	9·8	40·80
8·8	16·40	10·0	43·90

pH 8·45 to 12·77

Sörenson-Walbum (1920). 100 ml. mixtures of 0·1 M-glycine and 0·1 M-NaCl with 0·1 M-NaOH.

pH	x ml. glycine-NaCl	y ml. NaOH	pH	x ml. glycine-NaCl	y ml. NaOH
8·45	95	5	11·14	50	50
8·79	90	10	11·39	49	51
9·22	80	20	11·92	45	55
9·56	70	30	12·21	40	60
9·98	60	40	12·48	30	70
10·32	55	45	12·66	20	80
10·9	51	49	12·77	10	90

pH 2·62 to 9·16

Michaelis (1931). Stock solution : 9·714 g. sodium acetate $3H_2O$ + 14·714 g. sodium barbiturate in CO_2 free distilled water, made up to 500 ml. 5 ml. of this solution + 2 ml. of 8·5 per cent. NaCl (may be omitted) treated with x ml. of 0·1 N-HCl and (18 — x) ml. water.

x ml. 0·1 N-HCl	pH	x ml. 0·1 N-HCl	pH	x ml. 0·1 N-HCl	pH
16·0	2·62	9·0	4·93	4·0	7·66
15·0	3·20	8·0	5·32	3·0	7·90
14·0	3·62	7·0	6·12	2·0	8·18
13·0	3·88	6·5	6·75	1·0	8·55
12·0	4·13	6·0	6·99	0·75	8·68
11·0	4·33	5·5	7·25	0·5	8·9
10·0	4·66	5·0	7·42	0·25	9·16

pH 7·4 to 9·0

Holmes. Mixtures of M/5 boric acid with M/20 sodium tetraborate.

pH	M/5 H_3BO_3 (ml.)	M/20 $Na_2B_4O_7$ 10 H_2O (ml.)	pH	M/5 H_3BO_3 (ml.)	M/20 $Na_2B_4O_7$ 10 H_2O (ml.)
7·4	18	2	8·2	13	7
7·6	17	3	8·4	11	9
7·8	16	4	8·7	8	12
8·0	14	6	9·0	4	16

This buffer is recommended by Burtner and Lillie (1949) for making up Gomori's hexamine-silver solution (see Appendix 23).

pH 7·19 to 9·10

Gomori. 25 ml. of 0·2 M-tris(hydroxymethyl)aminomethane, Eastman-Kodak (M.W. 121·14), + x ml. 0·1 N-HCl and distilled water up to 100 ml.

pH	x ml. 0·1 N-HCl	pH	x ml. 0·1 N-HCl
7·19	45·0	8·23	22·5
7·36	42·5	8·32	20·0
7·54	40·0	8·41	17·5
7·66	37·5	8·51	15·0
7·77	35·0	8·62	12·5
7·87	32·5	8·74	10·0
7·96	30·0	8·92	7·5
8·05	27·5	9·10	5·0
8·14	25·0		

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APPENDIX 2

MAINTENANCE OF CRYOSTAT AND MICROTOME

(1) The working parts of the microtome should be greased with a silicone grease (Dow Corning) such as is used for high vacuum joints. Every 3 months or so it should be cleaned and regreased. No other attention is needed.

(2) A muslin bag containing 200 g. of self-indicating silica gel granules is kept in the chamber. In eastern England, where the average humidity is between 55 and 70 per cent., this needs changing at weekly intervals.*

(3) Under the conditions of humidity indicated above it may be necessary to defrost the cabinet about every 8 weeks. One small amount of ice which collects on the exposed refrigerating coils has no effect on their efficiency.

Storage of Tissues

Quenched or unquenched tissues can be stored in the cryostat cabinet at its normal maintenance temperature of — 15°. They must, however, be kept in airtight vessels in order to avoid the drying effect which is seen if they are merely kept in the interior of the cabinet itself. Tissues quenched in liquid air or in Arcton 6 (see Appendix 3, p. 785) cooled to — 158° with liquid air are best for storage. With these ice crystal growth takes place more slowly than with tissues from the cabinet itself. Tissues prepared in this way, or quenched at — 78°, can be maintained for very long periods without change in special cabinets maintained at — 70°. They must be protected against dehydration by wrapping them in some impervious material such as Saran wrap.

The gradual growth of ice crystals which takes place limits the acceptable time of storage for most tissues to something between 1 and 2 weeks at — 15°. Tissues like brain and liver suffer distortion in a much shorter time, and if sections of these are processed by mounting from the knife on to coverslips or slides, or by immediate delivery into a fixing bath, then the gross artifact is perpetuated. If they are used as free-floating sections in enzyme techniques, that is to say if they are allowed to thaw before fixation or mounting, then the histological appearances of the final product may be indistinguishable from those of sections cut from fresh blocks.

Technical Accessories

(1) **Brush.** A stiff paint brush of medium size hog bristle is essential both for dislodging sections which may have become stuck to the knife or to the anti-roll plate and for removing condensation from the knife surface. When " warm " coverslips or slides are used to pick up the sections this condensation may have to be removed between cutting of each section or pair of sections.

(2) **Coverslip Pick-up.** As stated in Chapter II, there are a number of ways in which the freshly cut section can be handled. If it is to be picked up on a coverslip (standard size $\frac{7}{8}$ inch square), or on a microscope slide, the provision of a suction coverslip pick-up is absolutely essential. This simple piece of apparatus, shown in the diagram (Fig. 242) below, consists of a round perforated sucker disc (A) attached to a capillary glass tube 9 inches

* Alternatively, an open tray of anhydrous $CaCl_2$ can be employed.

long, having at its other extremity an inclined side arm to which a rubber teat (B) is fixed. The end beyond the side arm should be solid.

Fig. 244. Coverslip Suction Pick-up.

Clean coverslips or slides are picked up by squeezing the teat between the right thumb and forefinger, expelling air from the capillary tube. The sucker disc is then applied to the coverslip or slide and the teat released. After picking up the section by pressing coverslip or slide firmly and steadily on to the surface of the knife (having previously swung down the fluon anti-roll plate), the coverslip or slide is grasped between the thumb and forefinger of the left hand. Momentary pressure on the teat will then release either.

Before cutting again the knife surface must be brushed to remove the ice-film. A long artist's hog-bristle brush is recommended.

(3) **Dry Ice Knife Cooler.** The standard pattern knives used with Cambridge Rocker microtomes have a conveniently long handle which projects from the side of the knife holder, to one side or other. A hemicylindrical box of brass or steel is provided with a slot to take the handle of the knife, and a reversible screw for maintaining its position (Fig. 9, p. 20). The screw is reversed when changing the box (and the knife) from one side of the holder to the other. The box is filled with powdered dry ice and moved in and out on the handle of the knife to adjust the temperature of the cutting zone to the desired temperature. One filling lasts 3 hours or more.

Rapid Technique for Diagnostic Biopsies

(1) Mount tissue block (preferably not larger than $2 \times 1{\cdot}5$ cm.) on the brass microtome chuck with a little water.

(2) Freeze in CO_2 jet and transfer to microtome.

(3) Cut two or three 4 to 8 μ sections and mount on "warm" coverslips.

(4) Fix for 1–2 minutes in absolute methanol, or in 5 per cent. acetic-ethanol.

(5) Stain one section in 1 per cent. aqueous methylene blue (30 seconds); rinse, blot, dehydrate in absolute alcohol, clear in xylene and mount.

(6) Stain another section in 1 per cent. Solochrome cyanine RS in 0·1 M-citric acid (pH 2·1) for 3 minutes. Wash in running water until differentiated and mount in glycerine jelly.

The first section should be available in less than 4 minutes from the time the tissue is received.

APPENDIX 3

PREPARATION OF TISSUES BY FREEZE-DRYING

THE scheme of events detailed below refers to the use of an apparatus where the drying tissues are carried in the bottom of a glass tube such as those illustrated in Fig. 11. No essential difference in procedure is observed when using dryers in which the tissues are carried in baskets or trays (Fig. 12; Fig. 15, lower two diagrams).

Quenching

(1) Small pieces of tissue, preferably not more than 2 mm. thick, are cut or torn from the original larger specimens.

(2) These are placed on narrow plates of copper or brass, or on thin strips of copper or aluminium foil.

(3) The plate or foil, with its mounted tissues, is plunged into a tube containing one of three alternatives : (1) *iso*pentane cooled to about — 160° with liquid nitrogen (there is no need to measure the exact temperature ; a point at which the *iso*pentane becomes noticeably more viscous is chosen) ; (2) propane cooled to — 185° (see Stephenson, 1954, on need for caution) ; (3) dichlorodifluoromethane (Arcton 6,* Freon 12 ; CCl_2F_2) cooled to — 158° with liquid air or O_2.

(4) The frozen tissues are dislodged from the plate or strip of metal with the tip of a metal rod.

(5) Excess coolant is poured off.

Drying

(6) The tube containing the tissues is attached to the manifold of the vacuum apparatus. If an isolating tap is provided (as in Fig. 18 for instance) the pump or pumps will already be running.

(7) The isolating tap is opened, or the pumps are started.

(8) The tube containing the tissues is quickly immersed in its covering Dewar flask containing acetone, ethyl oxalate, or any other suitable solvent cooled with dry ice to — 40° or slightly below.

(9) If a cold finger is provided this is filled with liquid nitrogen, oxygen or air.

(10) If a distant vapour trap is fitted, this is also filled with liquid nitrogen, oxygen or air.

(11) The cold finger, the distant trap and Dewar flask are replenished as often as necessary, but if drying continues for longer than 8 hours the first may be allowed to empty. The temperature of the cooling bath in the Dewar flask must be checked frequently.

(12) Provided that a vacuum better than 10^{-4} mm. Hg. is maintained, the drying time with a cold finger type of apparatus will not exceed 16 hours for small blocks.

(13) When drying is completed the diffusion pump (if employed) is turned off and allowed to cool. Air is then admitted to the manifold by means of a tap (T in Fig. 11) and the mechanical pump is stopped.

* I.C.I. Ltd.

Embedding

(14) The tube containing the tissues is removed from the apparatus and the dried blocks are quickly transferred to a vacuum embedding bath (400 mm. Hg for 5 minutes) containing molten paraffin wax (58°) or polyethylene glycol wax (50°). (Alternatively, the embedding media can be placed at the bottom of the drying chamber and melted by application of moderate heat while the tissues are still under low vacuum.)

Cutting and Mounting (Paraffin-embedded Tissues)

(15) Cut 5–8 μ sections and cut the ribbons into single sections or into short strips containing a few sections each.

(16) Flatten the sections on cool slides previously covered with a thin film of egg albumin. Firm pressure from a finger is sufficient.

(17) Warm the slide until the wax just melts, then allow to cool and flatten again with the finger while the section is still cooling. (Slides can be stored in racks in a desiccator at 4°, but if prolonged storage is required it is better to store blocks, well coated with paraffin at their cut surface, under similar conditions.)

Preparation for Examination, Staining, or for Histochemical Reactions

The various techniques which can be applied to mounted freeze-dried sections have been discussed in Chapter III. For routine purposes 24 hours fixation in absolute ethanol or methanol will be found suitable. The majority of histological techniques can be applied to material fixed in this way.

Methods for Freeze-substitution

(A) Glycol Technique of Blank, McCarthy and De Lamater

Method. (1) Cut fresh slices of tissue 2–3 mm. thick into cold propylene glycol at — 20°.

(2) After 2 or more hours transfer to a mixture of Carbowax 4000, 9 parts ; Carbowax 1500, 1 part, at 55°. Allow the wax to infiltrate for 2–3 hours and then prepare blocks.

This method was recommended by the original authors for lipid studies.

(B) Peyrot's Modification of Lison Technique for Glycogen

Method. (1) Immerse very small pieces of tissue briskly in a solution of absolute acetone saturated with dry ice (— 80°).

(2) Transfer the frozen pieces to Rossman's fluid maintained at — 60° to — 65° in a container of dry ice.

(3) After 24–36 hours allow the fluid to rise slowly to room temperature.

(4) Wash two or three times in absolute alcohol.

(5) Clear and embed in the usual manner.

(C) The Woods and Pollister Technique for Plant Tissues

Method. (1) Quench small pieces of tissue (less than 1 $mm.^3$) in *iso*-pentane at — 160°.

(2) Transfer quickly to absolute ethanol at — 41° to — 45° (dry ice/ethyl oxalate).

(3) After 3 days replace the cold ethanol with either 75 per cent. ethanol at — 40° or with 95 per cent. ethanol containing 0·3 ml. acetic acid per 100 ml., also at — 40°. After 30–60 minutes allow to rise to room temperature and maintain for the same period.

(4) Dehydrate in absolute alcohol and embed in the usual way.

APPENDIX 4

FIXATIVES

FORMALIN is the most widely used fixative in histological practice and the diagnostic histochemist will probably continue to receive a great deal of material already immersed in formalin-containing fluids. Since it is difficult to arrange to receive routine surgical and autopsy specimens in the fresh condition, and to freeze selected blocks in *iso*pentane cooled with liquid N_2 (— 180°) prior to storage at — 70°, it is necessary to be reconciled to the evils of formalin. After recent demonstrations of the excellent preservative qualities of formalin in respect of many common enzymes, this fixative is gradually returning to favour with histochemists although not with cytochemists, to whom all fixation is objectionable.

The bad qualities of formalin from the histological point of view are: (1) it allows considerable shrinkage during subsequent embedding ; (2) it forms black acid hæmatin (formalin) pigment in many tissues ; (3) it gives poor nuclear definition, especially in very cellular tissues ; and (4) it acts slowly. From the histochemical, as well as the histological point of view the first objection can be overcome by the use of frozen sections. The second objection can be overcome by maintaining the pH of the formalin solution in the region of neutrality and nothing but neutral or, preferably, buffered neutral formalin should be used for histochemical work. The fourth objection becomes in many cases a histochemical advantage, especially in enzyme work, but the third remains. The presence or absence of NaCl makes little difference. Below is given the buffered 10 per cent. neutral formalin of Lillie (1954) which is recommended for routine histochemical use :

40 per cent. formaldehyde . .	100 ml.
Distilled water	900 ml.
NaH_2PO_4, H_2O	4 g.
Na_2HPO_4 (anhydrous) . . .	6·5 g.

(Fixation time 24–72 hours.)

As an alternative I employ a variant of Baker's (1944) formol-calcium :

40 per cent. formaldehyde . .	150 ml.
1·3 per cent. calcium chloride * . .	850 ml.

(Fixation time 16–24 hours.)

The use of buffers may be avoided by using Amberlite IR-45 resin (Rohm and Hass Co., Philadelphia) either as a column or as a layer of resin at the bottom of the bottle.

Formalin-stable enzymes are well demonstrated in frozen sections from tissues fixed in this fixative at 4° for 16 hours. The following routine is used for paraffin embedding after cold formalin fixation :

(1) Rinse once in cold (0°–4°) distilled water and leave in excess cold distilled water for 24 hours.

(2) Begin dehydration in three changes of absolute acetone, each for 20 minutes at 0°–4°.

* Anhydrous, granular, technical grade.

(3) Continue dehydration in two changes of absolute acetone containing 1 per cent. Necol, 30 minutes in each, at 0°–4°.

(4) Transfer to 5 per cent. Necol in acetone, 30–60 minutes (0°–4°).

(5) Harden and clear in chloroform for 90 minutes, allowing the temperature to rise, at this stage, to that of the environment.

(6) Embed quickly in 56° paraffin wax *in vacuo* or, better, in one of the lower melting-point waxes or wax mixtures.

Fixatives for Special Purposes

Glycogen. With the possible exception of Bouin's fixative, the formalin-containing fixatives in routine use are all more or less objectionable from the histochemical point of view. Bouin's fluid remains one of the best routine histological fixatives for glycogen, but for histochemical purposes it should be superseded by Lison's " Gendre fluid."

Saturated picric acid in 96 per cent. alcohol	85 parts
40 per cent. formaldehyde	10 parts
Glacial acetic acid	5 parts

This fixative is cooled to — 73° before use (fixation time 18 hours), and the resulting localization of glycogen compares favourably with that shown by freeze-dried tissues. The weak Bouin fixative for use with Baker's acid hæmatein test is given in Appendix 11.

Nucleic acids. For routine studies of the nucleic acids in paraffin-embedded material Carnoy's fixative is recommended :

Ethyl alcohol	60 ml.
Chloroform	30 ml.
Glacial acetic acid	10 ml.

For smears and for freeze-dried sections fixation in absolute methanol is preferable.

An alternative to Carnoy, which gives excellent fixation without interfering with the action of nucleases, is Lillie's (1949) AAF (acetic alcohol formalin) :

40 per cent. formaldehyde	10 ml.
Acetic acid	5 ml.
Ethanol	85 ml.

(Fixation time 24 hours at 4°.)

Newcomer (1953) designed his *iso*propanol fixative especially for the preservation of chromosomes. It is excellent for this purpose and permits most histochemical reactions to be carried out. Feulgen hydrolysis is prolonged, however, to 10 minutes for plant tissues and to 20 minutes for animal tissues.

*Iso*propanol	6 parts
Propionic acid	3 parts
Light petroleum	1 part
Acetone	1 part
Dioxane	1 part

(Fixation time 12–24 hours.)

Mucopolysaccharides and Mucoproteins

There are several more or less satisfactory variants of the older lead subacetate mixtures for fixing acid mucopolysaccharides. Two can be recommended. They are Lillie's (1954) alcoholic lead nitrate and the ethanol-acetic-subacetate of Mota, Ferri, and Yoneda (1956). The latter was devised for and is particularly good for the fixation of mast-cell granules.

The formula for Lillie's fixative is as follows :

Lead nitrate	8 g.
40 per cent. formaldehyde	10 ml.
Water	10 ml.
Ethanol	80 ml.

(Fixation times 24 hours at room temperature ; 2–3 days at 4° ; 10–14 days at — 25°.)

Mota *et al.* formula is given below :

Lead subacetate	1 g.
Ethanol	50 ml.
Water	50 ml.
Acetic acid	0·5 ml.

(Fixation time 24 hours.)

The two fixatives for acid mucopolysaccharides introduced by Williams and Jackson are as follows :

(1)	40 per cent. formaldehyde	10 ml.
	Cetyl pyridinium chloride	0·5 g.
	Water	90 ml.

(Fixation time 48 hours.)

(2)	Ethanol	50 ml.
	5-aminoacridine hydrochloride	0·4 g.
	Water	50 ml.

(Fixation time 48 hours.)

Enzymes

As a procedure for the preservation of several groups of enzymes Wolman and Behar (1952) recommended the following routine :

(1) Fix thin slices in 5 per cent. acetic acid in absolute alcohol for 4 hours at 4°.

(2) Dehydrate in absolute alcohol for 16 hours at room temperature.

(3) Clear in two changes of benzene, 20 minutes each.

(4) Embed in three changes of 56° wax, 10–15 minutes in each under low vacuum.

Particularly for the preservation of β-glucuronidase, Fishman and Baker (1956) recommend :

Neutral formaldehyde (Merck)	20 ml.
Chloral hydrate	0·1 g.
Distilled water	80 ml.

(Fixation time 17–24 hours at 8°.)

Lipids

Baker's (1944) formol-calcium has already been mentioned (p. 64) as a general fixative which preserves phospholipids well without destroying more than a fraction of the various formol-stable enzyme systems.

If the preservation of enzymes is not required Elftman's (1957) fixative may be employed.

Mercuric chloride	5 g.
Potassium dichromate	2·5 g.
Water	100 ml.

(Fixation time 3 days at room temperature.)

Fixation with osmium tetroxide is seldom required in histochemistry. Mixtures containing an oxidant as well as OsO_4 serve to distinguish normal from degenerating myelin, and they preserve lipids and lipoproteins. Swank and Davenport's (1935) fixative is a suitable one :

1 per cent. potassium chlorate	60 ml.
40 per cent. formaldehyde	12 ml.
Acetic acid	1 ml.

(Fixation time 6–10 days.)

APPENDIX 5

THE MILLON REACTION (Bensley and Gersh Modification)

(Freeze-dried, formalin; alcohol, etc., paraffin sections)

Preparation of the Reagent

Add 400 ml. conc. nitric acid (Sp. G. 1·42) to 600 ml. distilled water and stand 48 hours. Dilute 1 : 9 with distilled water and saturate with crystalline mercuric nitrate. Filter and add to 400 ml. of the filtrate 3 ml. of the original diluted nitric acid and 1·4 g. sodium nitrite.

Method

(1) Remove wax from paraffin or freeze-dried sections with light petroleum.

(2) Rinse in absolute acetone and allow to dry in air.

(3) Cover section with Millon reagent and either stand at room temperature until the maximum colour develops or place in a covered Petri dish in the 60° incubator. The reaction proceeds slowly in the cold, but is complete in 30–60 minutes in the incubator.

(4) Rinse in cold or warm 2 per cent. nitric acid (2 ml. conc. acid Sp. G. 1·42, 98 ml. water).

(5) Dehydrate rapidly in 70 per cent. and absolute alcohol.

(6) Clear in xylene.

(7) Mount in Canada balsam.

Result (Fig. 28)

Proteins containing tyrosine stain orange to rose-red. The colour is stable for over 12 months.

MILLON REACTION (Baker Modification)

(Formalin; paraffin sections)

The original author advises celloidin embedding, but this is not necessary.

Preparation of the Reagent

Add 10 g. $HgSO_4$ to 100 ml. 10 per cent. H_2SO_4 and heat until dissolved. Make up to 200 ml. Add 0·5 ml. 0·25 per cent. $NaNO_2$.

Method

(1) Bring sections through 50 per cent. alcohol to water.

(2) Place sections in a small beaker containing the reagent and boil gently.

(3) Stop heating and allow the solutions to come to room temperature.

(4) Remove sections and wash three times in distilled water (2 minutes each wash).

(5) Mount in glycerine jelly, or

(6) Dehydrate, clear and mount in DPX or other suitable synthetic medium.

Result

Tyrosine-containing proteins are stained red, pink or yellowish-red.

MERCURY-BROMPHENOL BLUE METHOD FOR PROTEINS (after Bonhag)

(*Carnoy, formalin, etc.; avoid osmium*)

Preparation of Solution

Two alternatives have been employed : (1) 1 per cent. alcoholic bromphenol blue saturated with $HgCl_2$ and (2) 1 per cent. $HgCl_2$ and 0·05 per cent. bromphenol blue in 2 per cent. aqueous acetic acid. The second of these is recommended.

Method

(1) Bring paraffin sections to water.
(2) Stain in one of the two alternative solutions for 2 hours at room temperature.
(3) Rinse sections for 5 minutes in 0·5 per cent. acetic acid.
(4) Transfer sections directly into tertiary butyl alcohol.*
(5) Clear in xylene and mount in a suitable synthetic medium.

Result

Proteins are stained a deep clear blue colour.

MOREL-SISLEY DIAZOTIZATION METHOD FOR TYROSINE (Lillie, 1957b)

(*Formalin, etc., hot chloroform/methanol; paraffin sections*)

Method

(1) Bring paraffin sections to water.
(2) Nitrosate for 18 hours at 3° in a mixture containing 6·9 g. $NaNO_2$, 5·8 ml. acetic acid, distilled water to 100 ml.
(3) Rinse in three changes of ice-cold distilled water, 5 seconds in each.
(4) Treat for 1 hour at 3° with a mixture containing 1 g. S-acid, 1 g. KOH, 2 g. urea and 100 ml. 70 per cent. alcohol.†
(5) Wash in three changes of 0·1 N-HCl, 5 minutes in each.
(6) Wash in running water, 10 minutes.
(7) Dehydrate, clear and mount in neutral synthetic resin.

Result

Tyrosine-containing proteins stain purplish-red to pink.

ISOELECTRIC POINT BY MINIMUM SOLUBILITY (after Catchpole)

(*Freeze-dried, paraffin sections*)

Method

(1) Cut sections of paraffin-embedded freeze-dried material at 6–10 μ.
(2) Float out on warm mercury (40° or 58° wax).

* This step obviates the necessity for restoring the pH to the neutral point in order to produce the blue form of the dye.

† This part of the reaction should be carried out in the dark since the reaction product is light-sensitive.

(3) Attach section to slide and dewax with light petroleum.

(4) Dry in air.

(5) Cover with selected buffer solution, and place section in a closed Petri dish.

A 0·1 M-veronal acetate buffer in stages of about pH 0·25 can conveniently be used between pH 3·0 and pH 9·0. Alternatively, acetate buffer may be employed from pH 3·6 to 5·4, phosphate buffer from pH 5·4 to 8·0 and borate buffer from pH 8·0 to 10·0. In the case of these buffers 0·1-M solutions and steps of pH 0·2 should be used.

(6) After 1–2 hours in buffer at room temperature, wash with fresh buffer solution.

(7) Immerse section in absolute alcohol for 8–16 hours.*

(8) Bring section to water (except for Millon reaction) and stain by the chosen method.

The following methods are recommended :

Simple Proteins—Millon, coupled tetrazonium (pp. 76 and 90).

Histones—Arginine reaction (Baker) (p. 119).

Nucleoproteins—Feulgen reaction (p. 193) ; methyl green-pyronin (p. 205).

Mucoproteins and Mucopolysaccharide-protein Complexes—Periodic acid-Schiff (Hotchkiss) (p. 231).

ACID SOLOCHROME CYANINE METHOD FOR BASIC PROTEINS

(Freeze-dried or fresh frozen sections with brief acetic-ethanol fixation, formalin-fixed frozen and paraffin sections; Carnoy, Zenker, Bouin, etc.)

Method

(1) Bring paraffin sections to water.

(2) Stain in 0·2 per cent. solochrome cyanine R † in 1 per cent. ortho-phosphoric acid (pH 1·7) or in 0·1 M-citric acid (pH 2·1) for 5–20 minutes at room temperature.

(3) Wash in running *hot* water until the section changes from orange through red and begins to turn blue.

(4) Pass rapidly through 70 per cent. to absolute alcohol.

(5) Clear in xylene and mount in a suitable synthetic medium.

Result (Plate IIA)

Nuclear chromatin, dark steel blue.

Acidophil tissue components, various shades of red.

The blue staining of the nuclei is completely fast, but washing may remove too much of the red complex. This is easily restored by brief immersion in the staining solution, followed by a further brief wash in water.

NINHYDRIN-SCHIFF METHOD FOR PROTEIN-BOUND NH_2 (Yasuma and Itchikawa, 1953)

The original authors recommended fixation in Zenker, with and without acetic acid, or absolute alcohol. I prefer 85 per cent. ethanol, Carnoy or 5 per cent. acetic-ethanol. Alternatively, cold microtome sections may be treated directly with the reagent.

* When mucoproteins are involved a formalin-containing fixative is preferable since some materials of this class remain soluble in water after alcoholic fixatives. In the case of nucleoproteins, 10 per cent. acetic acid should be added to the alcohol.

† I.C.I. Ltd., Blackley, Manchester.

Method

(1) Bring paraffin sections to water.

(2) Treat sections with 0·5 per cent. ninhydrin * in absolute ethanol for 16–20 hours at 37°.

(3) Wash gently in running water for 2–5 minutes.

(4) Immerse sections in Schiff's reagent † for 15–25 minutes.

(5) Wash in running water for 10 minutes.

(6) Counterstain nuclei, if required, in Mayer's hæmalum, wash, and differentiate in 1 per cent. acid alcohol.

(7) Dehydrate, clear and mount in a suitable synthetic resin.

Result

Proteins stain pinkish-red to magenta if they contain a sufficient number of reactive NH_2 groups.

CHLORAMINE-T SCHIFF METHOD FOR PROTEIN-BOUND NH_2 (Chu, 1953 ; Burstone, 1955)

(*Carnoy, cold acetone, etc.*)

Method

(1) Bring paraffin sections to water.

(2) Treat with 1 per cent. aqueous chloramine-T ‡ (pH 7·5) for 6 hours at 37°.

(3) Wash briefly in distilled water.

(4) Treat with 5 per cent. aqueous sodium thiosulphate for 3 minutes.

(5) Rinse in distilled water and immerse in Schiff's reagent for 20–30 minutes.

(6) Rinse in 10 per cent. aqueous sodium bisulphite.

(7) Wash in tap water.

(8) Dehydrate in alcohols, clear in xylene and mount in DPX.

Result

Protein-bound NH_2 groups stain pink or reddish-magenta.

HYDROXYNAPHTHALDEHYDE METHOD FOR PROTEIN-BOUND NH_2 (Weiss, Tsou and Seligman, 1954)

(*Ethanol, Carnoy, acetone, and paraffin sections, are best for cytoplasmic NH_2. Nuclear NH_2 is most clearly demonstrated after Zenker fixation*)

Method

(1) Bring paraffin sections to water, avoiding thiosulphate and iodine treatment in the case of mercury-fixed tissues.

(2) Incubate sections for 1 hour at room temperature in a freshly prepared solution containing 20 mg. 3-hydroxy-2-naphthaldehyde in 20 ml. acetone to which 30 ml. 0·1 M-veronal acetate buffer at pH 8·5 has been added.

(3) Wash in three changes of distilled water for 5 minutes in each.

* 1·0 per cent. Alloxan may be used as an alternative.

† Full-strength Schiff reagent should be used ; either the Itikawa and Ogura (1954) or the de Tomasi (1936) variants are recommended (see Appendix 8, p. 000).

‡ 10 per cent. solutions of commercial preparations of sodium hypochlorite can be used in place of chloramine-T.

(4) Develop sections by placing them in 0·1 M-veronal acetate buffer at pH 7·4 and shaking 25 mg. tetrazotized diorthoanisidine (Fast blue B salt) on to the surface. This is then stirred into the solution and the colour reaches its maximum in 3–5 minutes.

(5) Wash in running tap water for 5 minutes.

(6) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic medium.

Result (Fig. 31)

A blue colour develops in sites containing many reactive NH_2 groups. Where these are more sparsely distributed the colour is red or pink.

DIACETYLBENZENE REACTION FOR PROTEIN-BOUND NH_2 (Voss, 1940 ; Wartenburg, 1956-57)

(*Various fixatives; paraffin sections*)

Method

(1) Paraffin sections, preferably mounted with egg albumin, should be brought to water in the usual way.

(2) Immerse sections for 5–10 minutes in 0·1 M-veronal acetate buffer (pH 8·2).

(3) Immerse in 2 per cent. *o*-diacetylbenzene * in 70 per cent. ethanol with an equal amount of 0·1 M-veronal acetate buffer (pH 8·2).† Leave for 30–60 minutes at room temperature.

(4) Wash briefly in buffer at pH 7·0 and in three changes of distilled water.

(5) Dehydrate in alcohols, clear in xylene and mount in Canada balsam or in a suitable synthetic medium.

Result

Protein-bound amino groups are shown by the presence of a reddish colour.

THE COUPLED TETRAZONIUM REACTION

(*Formalin, alcohol, freeze-dried; paraffin sections*)

Method

(1) Bring sections to water and remove mercury if necessary.

(2) Immerse in freshly tetrazotized benzidine ‡ at 4° for 15 minutes. Alternatively (Burstone, 1955) one can use a 0·2 per cent. aqueous solution of Fast blue B salt in tris buffer at pH 9·2, for 5 minutes at room temperature.

(3) Wash in water and in three changes of veronal acetate buffer at pH 9·2, for 2 minutes in each change.

* This can be prepared by oxidation of *o*-ethylbutophenone (Remschneider, 1947 ; Remschneider and Weygand, 1955).

† The buffer should be added shortly before the beginning of the incubation.

‡ To about 3 ml. of a 2 per cent. suspension of benzidine, in 2 N-HCl at 4°, add 8 drops of cold fresh 5 per cent. sodium nitrite. Agitate rapidly for 10 minutes. Add 1 ml. 5 per cent. cold ammonium sulphamate and about 10 ml. of cold saturated aqueous sodium carbonate. As effervescence ceases and the solution becomes alkaline, it changes to a clear dark yellow. Add water to 50 ml. This solution deteriorates rapidly even at 4° and should not be used for more than an hour after preparation. Stable tetrazoates have not proved satisfactory substitutes for the fresh solution.

(4) Immerse in a saturated (1 g. in 50 ml.) solution of H-acid in veronal acetate buffer at pH 9·2 for 15 minutes.
(5) Wash in water for 3 minutes.
(6) Dehydrate in alcohol.
(7) Clear in xylene.
(8) Mount in balsam or DPX.

Result (Figs. 50, 54 ; Plate Ic)

The majority of tissue components stain in shades of reddish brown. If the coupled tetrazonium reaction is preceded by mild heat and benzoylation positive structures now stain either in shades of deep reddish brown or, as in the case of collagen, in purplish red. These colours are quite stable (over 3 years).

The use of formalin-fixed sections is probably less advisable than alcohol-fixed sections for critical work. Aromatic hydrogen groups are irreversibly blocked by formalin (see Chapter IV, p. 56) but the tetrazonium reaction is not prevented by formalin fixation, even though it is reduced.

Benzoylation

(1) After removal of mercury sections are brought to water and thence to absolute alcohol.
(2) Sections from (1), or paraffin sections containing no mercury, are immersed in light petroleum (petroleum ether) for 3 minutes.
(3) Remove and allow to dry in air.
(4) *Controlled heat stage* (optional). Apply heat at 60° in an incubator, or at 80° on the warm stage, for 5–10 minutes, to the dry sections.
(5) Place the dry sections in 10 per cent. benzoyl chloride in dry pyridine * for 10–16 hours at room temperature. (The duration of benzoylation depends on the type of fixation employed ; it is shorter for alcoholic and longer for formalin-containing types.)
(6) Rinse in absolute acetone and
(7) Immerse in absolute alcohol.
(8) Take sections to water.
(9) Proceed to the coupled tetrazonium reaction, Stage 2.

Acetylation

Details as above but for Stage 5 substitute the following :

Immerse in 10 per cent. acetic anhydride in dry pyridine and heat for 4–8 hours at approximately 100°, under a reflux condenser (apparatus described in Chapter XI, p. 296).

Acetylation for reversal of the periodic acid-Schiff reaction is not carried out by this method (see Appendix 9, p. 832).

THE DINITROFLUOROBENZENE (DNFB) METHOD FOR TYROSINE, SH AND NH_2 (Danielli ; Burstone)

(Freeze-dried, alcohol, acetone, formalin, etc.)

Method

(1) Bring sections to absolute acetone or alcohol, remove and allow to dry in air.

* This solution lasts for about 1 month but rapidly loses strength if water is allowed to contaminate it. Pyridine should be dried by distillation over barium sulphide.

(2) Treat with a saturated solution of DNFB in 90 per cent. ethyl alcohol saturated with sodium bicarbonate, 2–16 hours, at room temperature.
(3) Wash in three changes of 90 per cent. alcohol, and finally in water.
(4) Treat with 5 per cent. sodium hydrosulphite, 30 minutes at 45°.
(5) Wash in water.
(6) Treat with cold (0°–4°) nitrous acid,* 30 minutes.
(7) Wash in water.
(8) Treat with a cold (0°–4°) saturated solution of H-acid in veronal acetate buffer at pH 9·4, 15 minutes.
(9) Wash in water, dehydrate in alcohols, clear in xylene, mount in balsam or DPX.

Result

The sites of DNFB in the tissues appear in reddish-purple. (See Chapter V, p. 86, regarding the specificity of this method for tyrosine.) For tyrosine alone Danielli suggested blocking NH_2 by nitrous acid treatment and SH by means of iodoacetamide, prior to Stage 2.

THE DMAB-NITRITE METHOD FOR TRYPTOPHAN (Adams, 1957)

The original author recommends short fixation (6 hours) in neutral formalin but states that up to 24 hours is acceptable. As alternatives, 1 per cent. trichloro-acetic-ethanol, 70 per cent. methanol or 10 per cent. aqueous sulphosalicylic acid may be employed. Sections should be mounted on albuminized slides or, preferably, on chromate gelatin, followed by 3–4 days in the 37° incubator. Fresh frozen (cold microtome) sections mounted on slides and air-dried are quite suitable.

Method

(1) Bring sections to absolute alcohol and allow them to become just dry in the air at room temperature.
Alternatively,
(2) Remove from alcohol and coat with a thin film of celloidin (0·25 per cent.) ; proceed to the next stage without drying.
(3) Immerse sections in 5 per cent. *p*-dimethylaminobenzaldehyde in conc. HCl † (S.G. 1·18) for 1 minute.
(4) Transfer to an approximately 1 per cent. solution of $NaNO_2$ in conc. HCl for a further minute.
(5) Wash for 30 seconds in tap water.
(6) Rinse in 1 per cent. acid-alcohol.
(7) Dehydrate, clear and mount.

Result (Fig. 32)

Tryptophan-containing proteins are shown in varying intensities of deep blue. Strong reactions occur in fibrin, fibrinoid, Paneth cell granules, peptic cell granules, zymogen granules, muscle, neurokeratin and in hair root sheaths.

* Add 1 vol. of freshly prepared 5 per cent. sodium nitrite to 4 vols. of 2 N-HCl.
† Keeps 3–4 weeks in a vaselined Coplin jar.

THE ROSINDOLE REACTION FOR INDOLES (Glenner, 1957)

(10 *per cent. calcium acetate-formalin for* 3–6 *hours*)

Method

(1) Bring sections to absolute alcohol.
(2) Dry in air for 30 seconds.
(3) Treat for 3 minutes at 25° in a solution containing 60 ml. perchloric acid, 34 ml. acetic acid, 1 ml. conc. HCl and 1 g. *p*-dimethylaminobenzaldehyde.
(4) Transfer to a solution containing 35 ml. acetic acid and 5 ml. conc. HCl with 0·5 g. $NaNO_2$ added just before use. Allow to stand for 1 minute.
(5) Wash three times in acetic acid and carry through 50 per cent. acetic-xylene to pure xylene.
(6) Mount in Permount or other suitable synthetic medium.

Result

Tryptophan-containing proteins appear deep blue. The depth of staining is usually less than that shown by the preceding reaction.

THE POST-COUPLED BENZYLIDENE REACTION FOR INDOLES (Glenner and Lillie, 1957)

(10 *per cent. calcium acetate formalin*, 3–6 *hours. Tissues fixed in* $HgCl_2$, *picrate or bichromate give a diminished reaction*)*

Method

(1) Bring paraffin sections to absolute alcohol.
(2) Dry in air for 30 seconds.
(3) Treat for 5 minutes at 25° in a mixture containing 1 g. *p*-dimethylaminobenzaldehyde, 10 ml. conc. HCl and 30 ml. acetic acid.
(4) Wash gently three times in acetic acid.
(5) Place sections in a Coplin jar containing 40 ml. acetic acid with the addition of 1 ml. of a fresh diazotate of S-acid (8-amino-1-naphthol-5-sulphonic acid).† Leave for 5 minutes at room temperature.
(6) Wash gently three times in acetic acid (1 minute in each).
(7) Clear in 50 per cent. acetic-xylene, 20 per cent. acetic-xylene and in three changes of pure xylene.
(8) Mount in a suitable synthetic medium.

Result (Fig. 33)

Indole derivatives appear pale to dark blue, depending on their local concentration.

THE XANTHYDROL REACTION FOR INDOLES (Lillie, 1957)

(10 *per cent. calcium acetate formalin*, 3, 24 *or* 48 *hours*)

Method

(1) Bring paraffin sections to absolute alcohol.
(2) Dry in air for 30 seconds.

* Poorly reactive tissues fixed in picrate can be unmasked by treatment with saturated lithium carbonate in 70 per cent. ethanol. Mercury blockade can be reversed in the same way, or by treatment with BAL or 5 per cent. thiosulphate (37°, 5 minutes).

† Add 240 mg. S-acid to 3 ml. N-HCl and 6 ml. distilled water. Cool to 4° and add 1 ml. N-$NaNO_2$. Stir at 4° for 15 minutes.

(3) Immerse sections for 5 minutes in 2·5 per cent. xanthydrol in a mixture of 90 ml. acetic acid and 10 ml. conc. HCl.

(4) Wash gently in acetic acid, three times.

(5) Clear in 50 per cent. acetic-xylene, 20 per cent. acetic-xylene and in three changes of pure xylene.

(6) Mount in a suitable synthetic medium.

Result

Indole-containing tissue components stain violet. Strong reactions are given by zymogen granules, and by stomach chief cells in some species. The colour is not stable and fades within a few months.

THE NAPHTHYL ETHYLENEDIAMINE REACTION FOR TRYPTOPHAN (Bruemmer, Carver and Thomas, 1957)

(*Carnoy, Zenker, formalin; paraffin sections*)

Method

All reactions, except where otherwise stated, are carried out at 0°–4°.

(1) Bring paraffin sections to ice-cold 50 per cent. ethanol.

(2) Place for 15 minutes in a mixture containing equal parts of 8 per cent. $NaNO_2$ and 6 N-HCl.

(3) Wash in two changes of distilled water, 5 minutes in each.

(4) Immerse for 15 minutes in a freshly prepared solution of 2 per cent. N-(1-naphthyl) ethylenediamine in 95 per cent. ethanol containing N-HCl.

(5) Dehydrate in 70 per cent. tertiary butyl alcohol to which has been added a few drops of conc. H_2SO_4.

(6) Continue dehydration at room temperature in absolute butyl alcohol containing H_2SO_4.

(7) Clear in two changes of xylene containing 2 ml. acetic acid per 100 ml.* (Room temperature.)

(8) Mount in a suitable synthetic medium.

Result

A purple colour develops in tryptophan-containing proteins.

THE SAKAGUCHI REACTION FOR ARGININE I (Baker's 1947 Modification)

(*Various; paraffin sections; freeze-dried, cold microtome*)

Baker himself advises the use of celloidin-covered paraffin sections fixed in Zenker, Bouin, Susa or formal sublimate.

Method

(1) (Optional.) Remove wax and cover with a thin film of 1 per cent. celloidin ; harden.

(2) Bring sections to water.

(3) Remove from water and flick off till nearly dry.

(4) Flood with α-naphthol hypochlorite solution,† 15 minutes.

* If an orange-red colour is preferred to purple, the acid is omitted at this stage.

† Mix 2 ml. 1 per cent. NaOH, 2 drops of 1 per cent. α-naphthol in 70 per cent. alcohol, and 4 drops of 1 per cent. Milton (proprietary brand of stable sodium hypochlorite containing about 1 g. of NaOCl and 18 g. NaCl per 100 ml.).

(5) Drain and blot.
(6) Immerse in a mixture of equal parts of dry pyridine and chloroform.
(7) Mount in pyridine-chloroform, or in dry pyridine.

Result (Fig. 44)

Arginine-containing proteins appear in various shades of orange-red.

THE SAKAGUCHI OXINE REACTION FOR ARGININE II (Carver, Brown and Thomas' 1953 Modification)

(*Bouin, formalin, Carnoy; paraffin sections*)

Method

(1) Bring paraffin sections to 70 per cent. alcohol.
(2) Immerse sections in 0·3 per cent. 8-hydroxyquinoline in 30 per cent. ethanol for 15 minutes at room temperature.
(3) Quickly transfer the slide, without draining, into alkaline hypochlorite solution.* Leave for 60 seconds without movement.
(4) Transfer quickly, without draining, into alkaline urea solution.† Move the slide gently for 10 seconds, then transfer to a fresh alkaline urea bath for 2 minutes.
(5) Place slide in tertiary butyl alcohol for 4 minutes.
(6) Place in aniline oil for 3 minutes.
(7) Wash in xylene, 10 seconds.
(8) Mount in Permount or other synthetic resin containing 0·025 ml. aniline per 100 ml.

Result

An orange colour indicates the presence of arginine.

BLOCKING AND CONVERSION METHODS FOR PROTEIN END GROUPS

Deamination (NH_2)

Nitrous acid is used at 0°–4° in various mixtures. At this temperature 18–48 hours in a mixture of equal parts of 10 per cent. acetic acid and 5 per cent. sodium nitrite is usually sufficient. Alternative mixtures using HCl (equal parts of 8 per cent. $NaNO_2$ and 6 N-HCl) are slower in action than acetic acid mixtures as far as tissues are concerned.

Dinitrophenylation (α-NH_2, ϵ-NH_2, tyrosyl-OH, SH, imidazole)

Treat sections with 1 per cent. 2,4-dinitrofluorobenzene in 90 per cent. ethanol containing 0·01 M-NaOH, for 16–20 hours at room temperature.

Acetylation. See p. 796

* Standardize a fresh commercial preparation of sodium hypochlorite (Milton, Chlorox) against 0·1 N-sodium thiosulphate. Use for the titration 1 ml. of hypochlorite, 5 ml. N-potassium iodide, 8 ml. conc. HCl and 50 ml. water. (The average chlorine content of fresh commercial hypochlorite is 1·6 N). The fresh hypochlorite is used as a stock solution. Prepare a 0·15 N stock solution of KOH.

For use, measure appropriate quantities of the two stock solutions into a 100 ml. cylinder, to make a final concentration of 0·15 N-chlorine and 0·015 N-KOH.

† To 10 ml. 0·15 N-KOH in a 100 ml. cylinder add 15 g. urea. Dilute with water to 25–30 ml. and mix until the urea is dissolved. Add 70 ml. tertiary butyl alcohol, dilute to volume or mix.

Benzoylation. See p. 796

Nitrobenzoylation (NH_2, tyrosyl-OH)

Treat sections with 5 per cent. *p*-nitrobenzoyl chloride in dry pyridine for 4 hours at 37°.

Methylation (COOH)

For most tissues treatment with 0·1 N-HCl in absolute methanol (1 per cent. HCl), for 2–3 days at 20°–25°, is sufficient to block carbonyl groups completely.

Demethylation

Reversal of methylation is only possible to a limited extent in the case of the majority of tissue components. Treatment for 20 minutes with 0·5 per cent. potassium permanganate is followed by a brief rinse in 2 per cent. oxalic acid.

Performic Acid Oxidation (Tryptophan, Cystine)

Prepare fresh performic acid by adding 4 ml. of 30 per cent. H_2O_2 (100 vol.) and 0·5 ml. conc. H_2SO_4 to 40 ml. 98 per cent. formic acid. The peroxide must be reasonably fresh and stocks older than 2–3 weeks should not be used. The performic acid solution should be degassed just before use by vigorous stirring with a glass rod and used within 8 hours.

Oxidation for 15–60 minutes at room temperature is normally employed.

Peracetic Acid Oxidation (Tryptophan, Cystine)

Peracetic acid (40 per cent.) is usually available commercially.* If it is not, it can be made by adding 5 ml. 30 per cent. H_2O_2 to 10 ml. acetic anhydride. After thorough mixing the reagent is allowed to stand for 2 hours, and diluted with an equal quantity of distilled water before use. Alternatively, add 9·5 ml. acetic acid and 0·25 ml. conc. H_2SO_4 to 30 ml. H_2O_2. Stand for 1–3 days before use and store at 0°–4° (months). Commercial (40 per cent.) peracetic acid is best employed as a 2 or 4 times dilution with distilled water, and tissues are oxidized for 2–4 hours.

Permolybdic and Pertungstic Acids

These acids are made by adding to 60 ml. water, 30 g. orthophosphoric acid (H_3PO_4) and 3 ml. 30 per cent. H_2O_2, 0·5 g. of either ammonium molybdate or sodium tungstate. Oxidize tissue sections at room temperature for 4–16 hours.

Persulphate Oxidation (tryptophan)

A fresh alkaline solution of persulphate is prepared by dissolving 1 g. potassium persulphate ($K_2S_2O_8$) in 40 ml. 0·5 N-KOH. Sections are incubated for 12–18 hours at 20°–22°.

Iodination (tyrosine, possibly tryptophan)

Treat sections for 24 hours at room temperature with a mixture of 30 ml. Gram's iodine (iodine, 1 g. ; KI, 2 g. ; water, 300 ml.) and 2 ml. 3 per cent. ammonia. The pH of this solution should be approximately 10. Alterna-

* From Laporte Chemicals Ltd., Luton, Beds.

tively, 0·78 N-I_2 in ethanol may be used for 72 hours at 25°. Tryptophan is not iodinated by this last procedure.

Iodine Oxidation (SH)

Treat sections for 4 hours at room temperature with an aqueous solution containing 38 mg. iodine and 33 mg. potassium iodide per 100 ml. This solution should be titrated to pH 3·2 with 0·01 N-HCl before use.

Iodoacetate Block (SH)

Treat sections for 20 hours at 37° with 0·1 M-iodoacetic acid titrated to pH 8 with 0·1 N-NaOH.

Maleimide Block (SH)

Treat sections for 4 hours at 37° with 0·1 M-N-ethyl maleimide in 0·1 M-phosphate buffer at pH 7·4. Follow this by washing in 1 per cent. acetic acid and then in tap-water.

Mercaptide Block (SH)

The most commonly used reagents for this purpose are mercuric chloride and phenylmercuric acetate. The latter has been used as a 0·001 M-solution in *n*-propanol (72 hours, 20°). Tolyl mercuric chloride and methylmercury iodide have also been used at the same concentration. Many other organic mercurials can be employed. All require long periods for complete reaction, and the resulting mercaptides break down easily in some cases so that the blocked SH groups become free to react.

Thioglycollate Reduction (SS)

Cover sections with a thin layer of 0·5 per cent. celloidin. Incubate for 4 hours at 37° in 0·5 M-thioglycollic acid (freshly made) titrated to pH 8 with 0·1 N-NaOH. Afterwards wash in tap-water, rinse briefly in 1 per cent. acetic acid and wash again in water.

THE NITROPRUSSIDE REACTION FOR SH (Hammett and Chapman)

(*Fresh slices*, 40–100 μ)

Method

(1) Place the section on a dry watch-glass.

(2) Cover with a small amount of distilled water (say 0·25 ml.).

(3) Add 0·05 ml. 27–29 per cent. ammonium hydroxide and 0·05 ml. 1 per cent. sodium nitroprusside.*

(4) Displace section to one side and underline with 0·25 g. crystalline ammonium sulphate.

Result

A pink colour develops which fades after a short period. It is possible to distinguish between areas containing many or few SH groups and, with practice, to estimate roughly the amount of SH present.

* Fresh solutions must be used. No solution should be more than 1 hour old.

THE FERRIC FERRICYANIDE METHOD FOR SH (Chèvremont and Fréderic, 1943)

(Formalin-frozen sections; various, including fresh sections)

The authors recommend short fixation, of a few hours only, and frozen or paraffin sections. In the case of the latter the time occupied in the embedding process should be as short as possible.

Reagent

One per cent. ferric sulphate, $Fe_2(SO_4)_3$, 3 parts, added to a freshly prepared 0·1 per cent. potassium ferricyanide, $K_3Fe(CN)_6$, 1 part. The pH should be adjusted to 2·4.

Method

(1) Wash sections in distilled water.

(2) Immerse in three changes of the ferricyanide reagent for a total of 10–20 minutes (frozen sections) or 20–25 minutes (paraffin sections).

(3) Wash in distilled water.

(4) Counterstain nuclei with carmalum 6–18 hours (optional).

(5) In the case of frozen sections, mount in glychrogel. With paraffin sections, dehydrate in alcohols, clear in xylene, and mount in DPX.

If the method is being used as a critical histochemical test, differentiation is not permissible. It is possible, however, by brief rinsing in 2 per cent. alkaline alcohol (NaOH in 60 per cent. alcohol), to reduce the diffuse blue staining of background structures and so to emphasize the strongly positive SH-containing elements.

Control Sections

Yao (1949) suggested the use of control sections treated with saturated aqueous $HgCl_2$ for 1 hour. Such sections should give a negative result in all areas where the reduction of ferricyanide is due to sulphydryl. In practice neither the mercaptan-forming agent nor the time of treatment are satisfactory, particularly for frozen sections. In the latter sulphydryls continue to react freely and, in addition, reactive groups are formed by the oxidation of lipid substances. The use of saturated phenyl mercuric chloride in butanol, for 2–3 days, is recommended instead of saturated aqueous mercuric chloride, but malcimide blocking is usually preferable.

Result (Fig. 35). Sulphydryls blue, nuclei red.

THE RSR (MERCURY ORANGE) METHOD FOR SH (Bennett, 1951)

(Trichloroacetic-teased; freeze-substituted, butanol; freeze-dried; cold microtome, etc.)

Preparation of the Reagent

(1-(4-chloromercuriphenylazo)-2-naphthol. Called red sulphydryl reagent, RSR.)

(1) Prepare *p*-aminophenylmercuric acetate by the method of Dimroth (*Ber. dtsch. chem. Ges.*, **35**, 2032, 1902).

This involves the direct mercuration of aniline which is performed as follows :

Add 31·8 g. mercuric acetate in 160 ml. of water to 18·6 g. of freshly distilled aniline. After standing for a short time the pale yellow prisms of the *p*-isomer begin to separate ; after 3 hours the supernatant is decanted and the crystals are washed with water.

(2) Diazotize 35·4 g. of *p*-aminophenyl mercuric acetate, in 500 ml. 50 per cent. acetic acid with 7·0 g. of sodium nitrite, at — 5°.

(3) Filter off the diazonium salt and couple with 2-naphthol (15 g. of 2-naphthol, 180 g. of NaOH in 2 litres of iced water).

(4) Stand for a few hours, filter and wash precipitate, dissolve in 200 ml. glacial acetic acid, filter and reprecipitate by dilution to 2 litres.

(5) Collect precipitate, wash and dissolve by refluxing with 3 litres of ethanol in a water bath.

(6) Filter the hot solution, bring to boil under a reflux condenser and add 5·8 g. NaCl in 150 ml. of 60 per cent. ethanol.

(7) Reflux for 30 minutes.

(8) Collect the cottony red precipitate by hot filtration and wash several times with hot 50 per cent. ethanol.

(9) Recrystallize three times from *n*-butanol. (Yield 3·6 g.)

Solvents for the Reagent

A variety of solvents can be used for RSR and the number of sulphydryl groups which react depends on the particular solvent used. Bennett originally recommended toluene, *n*-butanol or *n*-propanol, but later suggested that from six to eight times the number of SH groups would react if RSR was dissolved in dimethylformamide. Mescon and Flesch used 80–100 per cent. ethanol, and in some early studies with the method I used the same solvent. There is some evidence, however, that the increase in colour by comparison with RSR in other solvents is due to non-specific staining. For some time past I have used dimethyl formamide exclusively, and unfixed cold microtome sections mounted on slides or coverslips.

Method (according to Bennett, with modifications)

(1) Fix in 5 per cent. trichloroacetic acid and follow by washing in distilled water and dehydration in various strengths of ethanol. Immerse in *n*-propanol or *n*-butanol and tease into small fragments.

Alternatively,

(2) Freeze small strips of tissue in *iso*pentane cooled to about — 160° with liquid N_2 and follow by immersing the frozen tissue in *n*-propanol or *n*-butanol at —20°. Allow to remain for 10–12 days with occasional changes of solvent at — 20°. Finally, tease into small fragments for study.

(3) Place the fragments obtained by either of the above manœuvres in a saturated solution of RSR in butanol (about $1{\cdot}25 \times 10^{-5}$ M), propanol (about $5{\cdot}6 \times 10^{-6}$ M), or dimethyl formamide (5×10^{-5} M). Leave for 16–48 hours (until no further uptake of RSR occurs).

(4) Wash in several changes of pure solvent (propanol or butanol).

(5) Clear in xylene.

(6) Mount in clarite.

No fading occurs unless mercaptans are present in the mounting medium.

For formalin-fixed paraffin sections or for unfixed freeze-dried and cold microtome sections, proceed as follows :

(1) Bring sections to absolute alcohol (not cold microtome sections).

(2) Immerse in a saturated solution of RSR in dimethylformamide for 16–48 hours.

(3) Wash twice in absolute alcohol.
(4) Clear in xylene.
(5) Mount in DPX.

For formalin-fixed frozen sections proceed as follows :

(1) Bring sections from water to 75 per cent. alcohol.
(2) Immerse in saturated BSR in dimethylformamide, 16–48 hours.
(3) Wash twice in 75 per cent. alcohol.
(4) Bring to 50 per cent. alcohol and thence to water.
(5) Mount in glycerine jelly.

Result (Fig. 34)

The colour of SH-containing structures varies between pale orange and a darker orange-red.

Control Sections

Where there is any doubt as to the specificity of the reaction observed with RSR, control sections should be treated for 48–72 hours with a saturated solution of phenyl mercuric chloride in butanol or alcohol, or with 0·1 M-N-ethyl maleimide (4 hours, 37°).

THE PERFORMIC ACID-SCHIFF METHOD (Pearse, 1951)

(*Various; paraffin sections*)

Reagents

Performic Acid. Add to 40 ml. of 98 per cent. formic acid 4 ml. of 30 per cent. (100 vol.) H_2O_2* and 0·5 ml. conc. H_2SO_4. Allow the mixture to stand for at least an hour before using, and use preferably within 24 hours.

Schiff's Reagent. The de Tomasi or Itikawa preparations are preferred to the Barger and DeLamater modification for the purpose of this method (see Appendix 8, p. 822).

Method

(1) Bring paraffin sections to water with removal of mercury precipitate where necessary. (Celloidin-coating may be employed if sections fail to remain adherent to the slides.)
(2) Treat with performic acid solution for 10–30 minutes.
(3) Wash in water 2–5 minutes.
(4) Immerse in Schiff's solution for 30–60 minutes.
(5) Wash in warm running water for 10 minutes.
(6) Dehydrate in the alcohols, clear in xylene, mount in DPX.

Result (Fig. 36)

Keratin-containing structures appear bright pink to deep magenta and the amount of colour reflects the amount of available SS groups. Since the mechanism of the reaction is doubtful, however, it should not be used, at present, in the applied histochemistry of SS.

* Use fresh 100 vol. H_2O_2, preferably not more than 3 weeks old after opening the bottle.

THIOGLYCOLLATE-FERRIC FERRICYANIDE FOR SS (Adams, 1956)

(*Trichloroacetic-ethanol, formol-calcium, formalin, etc.*)

Method

(1) Bring paired sections to water.

(2) Immerse both sections for 30 minutes in 2·5 per cent. aqueous sodium thioglycollate adjusted to pH 8·0 (green to 9-naphthol-phthalein).

(3) Wash in weakly acidified distilled water (pH 4·0) for 3 minutes.

(4) Wash in running tap-water for 3 minutes, and rinse in distilled water.

(5) Transfer the second (control) slide to phenyl mercuric chloride in butanol (48 hours) or to 0·1 M-N-ethyl maleimide (4 hours, 37°). After blocking, bring again to water.

(6) Both sections should now be immersed in a fresh * solution containing 10 ml. freshly made 1 per cent. potassium ferrocyanide and 30 ml. 1 per cent. ferric chloride.† Leave for 1½ minutes.

(7) Wash in three changes of distilled water for 10 minutes.

(8) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic medium.

Result

Disulphides (stratum corneum, posterior pituitary neurosecretory material, etc.) stain Prussian blue. Any green colour may be tentatively interpreted as indicating weak reducing groups.

PERFORMIC ACID-ALCIAN BLUE METHOD FOR SS GROUPS (Adams and Sloper, 1955, 1956)

(*Formalin, formol-calcium, etc.; paraffin sections*)

Preparation of Reagents

Performic Acid. Prepared as described for the performic acid-Schiff method.

Alcian Blue. Three per cent. (w/v) in 2 N-sulphuric acid. Dissolve the dye by heating to 70° and filter when cool (pH 0·2–0·3).

Method

(1) Bring paraffin sections to water.

(2) Blot gently to remove excess water.

(3) Immerse in the performic acid reagent for 5 minutes after vigorous stirring to remove dissolved gas.

(4) Wash gently in tap-water for 10 minutes.

(5) Rinse in 70 per cent. and absolute alcohol, blot with filter paper to flatten the creases and again rinse in tap-water.

(6) Warm at 50°–60° until the section is just dry.

(7) Rinse again in absolute alcohol and finally in tap water for 1 minute.

(8) Stain for 1 hour at room temperature in the acid Alcian blue mixture.

(9) Wash for 5 minutes in tap-water.

(10) Counterstain can be applied at this point, if required.

(11) Dehydrate, clear and mount in a suitable synthetic medium.

* Not more than 5 minutes old.

† Filter before use.

Result (Fig. 39 ; Plate II)

Structures containing 4 per cent. or more of cystine appear dark steely blue in colour. Lesser amounts of cystine appear pale blue.

THE ALKALINE TETRAZOLIUM REACTION (SS AND SH)

(*Formalin, trichloroacetic-ethanol, etc.; paraffin sections*)

Method

(1) Bring sections to water.

(2) Incubate at 56°–60° in a solution of blue tetrazolium salt, in glycine buffer at pH 12–12·5,*† for 60–90 minutes.

(3) Wash well in running cold water.

(4) Mount in glycerine jelly.

Result (Fig. 40)

Thickly distributed protein-bound (i.e. non-lipid) reducing groups appear dark blue. Lipid reducing groups appear red. Weaker reduction (sparsely distributed reducing groups) gives a reddish colour in all cases. If the tetrazolium mixture of Barrnett and Seligman is used most of the colour developed can be ascribed to SS and SH groups.

THE DDD REACTION FOR SH GROUPS (Barrnett and Seligman, 1952)

(*Trichloroacetic-ethanol, Carnoy, Bouin, formalin, etc.; paraffin sections*)
(*Unfixed, or trichloroacetic-ethanol fixed, cold microtome sections*)

Method

(1) Bring paraffin sections to water.

(2) Incubate for 1 hour at 50° in a solution containing 35 ml. 0·1 M-veronal acetate buffer (pH 8·5) and 15 ml. absolute ethanol in which has been dissolved 25 mg. of the DDD reagent. Some of the reagent may remain in suspension.

(3) Cool to room temperature.

(4) Rinse sections briefly in distilled water.

(5) Wash for 10 minutes in two changes of distilled water acidified to pH 4–4·5 with acetic acid. This step converts the sodium salt of the reagent, and of the unwanted reaction product (6-thio-2-naphthol), to free naphthols.

(6) Extract the free naphthols by passage through a graded series of alcohols and wash twice in absolute ether for 5 minutes in each wash.

(7) Rinse in distilled water.

(8) Stain for 2 minutes at room temperature in a freshly prepared solution of 50 mg. tetrazotized diorthoanisidine (Fast blue B salt) ‡ in 50 ml. 0·1 M-phosphate buffer at pH 7·4.

(9) Wash in running tap-water.

(10) Dehydrate in alcohols, clear in xylene and mount in Permount, DPX, or other suitable medium.

* Dissolve 10 mg. of tetrazolium salt in 40 ml. 0·1 M-glycine buffer at the required pH, heating to 70° and stirring. Filter while hot.

† Barrnett and Seligman (1956) used 25 mg. blue tetrazolium in 48 ml. aqueous KCN with 2 ml. NaOH, and incubated sections for 8–12 hours.

‡ Alternatively a mono-coupling diazotate such as Fast blue salt K may be used.

Result (Figs. 41 and 42)

Blue staining indicates a high concentration of SH groups ; red staining areas contain lower concentrations.

THE DDD REACTION FOR SH AND SS GROUPS

If the reaction is required to demonstrate SH and SS groups together sections are first treated with thioglycollate (see p. 802). If SS groups alone are required it is necessary to block existing SH groups with iodoacetate or N-ethyl maleimide (see p. 802) and then to reduce the SS groups to SH with some reagent which will not unblock the original SH groups. Potassium cyanide is suitable for this purpose.

THE NAPHTHYL MALEIMIDE (HNI) REACTION FOR SH (after Seligman, Tsou and Barrnett, 1954)

(*Trichloroacetic-ethanol, paraffin sections. Cold microtome sections, unfixed or TCA-fixed*)

Preparation of Solutions

HNI. Dissolve 25–50 mg. N-(4-hydroxy-1-naphthyl)*iso*-maleimide in 40 ml. dimethylformamide.

Diazonium Salts. Stir 10–20 mg. of the chosen stable diazotate into 40 ml. 0·1 M-Tris buffer (pH 10). Filter through Whatman No. 40 paper and use at once.

Method

(1) Bring paraffin sections or mounted fresh frozen sections to alcohol.
(2) Incubate in the HNI solution for 4–16 hours at 37°.
(3) Bring through 70 per cent. alcohol to water.
(4) Treat with the alkaline diazotate for 5–10 minutes at room temperature.
(5) Wash in running water.
(6) Dehydrate in alcohols, clear in xylene and mount in Fluormount * or other non-fluorescent medium.

Result (Fig. 43)

By visible light proteins containing reactive SH groups appear reddish-purple, occasionally blue if the concentration is sufficiently high. Under ultra-violet light a brilliant deep red fluorescence indicates the presence of SH in regions in which it is not visible with the light microscope.

The results obtained with a variety of diazonium salts are given in the table below.

* Obtainable from G. T. Gurr Ltd., London, S.W.6.

Results with HNI and various Diazotates at pH 10·0

No.*	Diazotate from :	Visible light	U.V. light	Coupling time (mins.)
2	4-benzoylamino-2 : 5-dimethoxyaniline	Purple	Red (contrast +)	3
6	*o*-dianisidine	Red	Brilliant red (contrast +)	20
7	4-chloro-*o*-anisidine	Brick red	Orange-red	20
9	5-chloro-*o*-toluidine	Orange-red (contrast +)	Orange	4
12	3-nitro-*p*-anisidine	Red (contrast +)	Fiery red	15
15	4-amino-4′methoxy diphenylamine .	Brown	Pinkish-red	5
16	3-nitro-*o*-toluidine	Brown	Pinkish-red	5
18	4-amino-3,1′dimethyl azobenzene .	Red	Nil	5

For visible light observations I have found Salts 2, 6, 9, 12 and 18 to be excellent, while for fluorescence observations Salts 2, 6, 7, 9 and 12 are good. The relative intensity with which identical structures are stained varies with the salt used for coupling, and this suggests that stereochemical factors are operating. In doubtful cases two or more different salts should be used on serial sections.

* See list of diazotates on p. 870.

APPENDIX 6

PREPARATION OF FLUORESCEIN-4-ISOCYANATE (Coons and Kaplan, 1950 ; de Repentigny and James, 1954)

Preparation of Nitrofluorescein

100 g. 4-nitrophthalic acid and 100 g. resorcinol are intimately mixed in a beaker and heated on an oil bath at 195°–200° until the mass is dry (12 hours). When cool, the melt is chipped from the beaker, ground in a mortar, and boiled with 1,600 ml. 0·6 N-HCl for 1 hour. After washing by decantation with 3 × 300 ml. hot HCl solution it is collected on a Buchner funnel. The resulting brown paste is washed with 5 litres of water and dried.

Reduction to Aminofluorescein

Suspend 2 g. crude nitrofluorescein in 100 ml. absolute ethanol and shake with 1·5 g. Raney nickel in an atmosphere of hydrogen at room temperature and pressure. After 90 minutes remove the nickel by centrifugation, wash with 15 ml. ethanol and add the washings to the main lot. Dilute with an equal quantity of water and allow to stand. Colourless needles form which are separated by filtration. The two isomers, aminofluorescein I and aminofluorescein II, are present.

Separation of the Isomers by Chromatography

Coons and Kaplan separated the isomers by fractional crystallization of nitrofluorescein diacetates. This method is laborious and, provided that small quantities (up to 400 mg.) are acceptable, the alternative method of separation of the two aminofluoresceins by chromatography is preferable.

Stationary Phase. Kieselguhr (Hyflo-Supercel) column with 0·2 M-phosphate buffer at pH 8·0 (3 ml. buffer to 5 ml. kieselguhr).

Mobile Phase. A mixture of *n*-butanol and *cyclo*hexane ; de Repentigny and James recommend the attachment of an automatic fraction collector to a column 70 × 3 cm., containing 100 g. kieselguhr.

Preparation of Fluorescein Isocyanate

The required amount (10–60 mg.) of isomer I or isomer II, or of a mixture of the two if the pure isomers are not available, is added to 5 ml. of dry acetone. This is added through a dropping funnel to 15 ml. acetone previously saturated with phosgene. As each drop of the amine solution enters the flask a yellow precipitate forms and dissolves. Allow the reaction to proceed for a further 30 minutes after adding all the amine. Then remove the flask from the phosgene train and add some anthracite chips (to prevent bumping). Take the solution to dryness *in vacuo* over a water bath at 45°. Dissolve the resulting greenish-brown gum in 2 ml. acetone and 1 ml. dioxan and use this solution for adding to the chilled protein solution.

Preparation of Fluorescein Conjugates

A 50 ml. flask fitted with a mechanical stirrer is fixed in an ice-bath and into it are put 10·5 ml. saline (0·15 M-NaCl), 3 ml. carbonate-bicarbonate buffer (0·5 M, pH 9·0), 2·5 ml. dioxan and 0·5 ml. acetone. When the temperature reaches 0°–2° add the protein-containing solution (300–400 mg.,

50–100 mg. per ml.) and then, drop by drop, the required amount of *iso*cyanate, calculated as amine. Stir for 18 hours in the cold. Transfer the solution to a cellophane bag and dialyse against buffered saline until the fluorescence of the dialysate falls below that of a 1/20,000,000 solution of fluorescein (5–6 days). Remove from the bag, centrifuge to remove precipitate, and store at 4°.

PRESERVATION OF FLUORESCEIN ISOCYANATE (Goldman and Carver, 1957)

Although sealed ampoules containing the acetone solution keep for " several months " they are not really convenient either for storage or use.

Goldman and Carver suggest the following procedure :

(1) Dissolve 20 mg. per ml. of the *iso*cyanate in acetone-dioxan.*

(2) Place 1 ml. in a flat dish and soak up with a 20 mm. square of chromatographic paper.

(3) Dry in front of a fan (*N.B.*—dioxan is toxic) and store over $CaCl_2$ on a desiccator.

(4) For labelling. Cut a strip of the paper to give the calculated amount of *iso*cyanate and add to the protein solution with enough 0·5 M-carbonate to produce pH 9·0. The volume of buffer should be about 10 per cent. of that of the protein solution.

With this procedure denaturation of protein is avoided.

PREPARATION OF ACETONE-DRIED TISSUE POWDERS FOR ABSORPTION OF FLUORESCEIN CONJUGATES (Coons, Leduc and Connolly)

Place 25–50 gm. of fresh or frozen tissue in a Waring blendor with an equal volume of 0·15 M-NaCl solution. Homogenize with short repeated runs to avoid heating. Pour the homogenate into a beaker and add 4 volumes of acetone, with stirring. Allow to stand for a few minutes, decant and discard the supernatant. Pack the precipitate by centrifugation, wash in the centrifuge with several changes of saline until the supernatant is free from hæmoglobin. Suspend the washed precipitate in an amount of saline about equal to the volume of the precipitate and add 4 vols. acetone and harvest on a Buchner funnel. Wash the precipitate with acetone and allow to dry on the funnel. Dry overnight at 37° and store at 4° in stoppered containers.

Absorption of Fluorescein Conjugates

For use, stir the powder obtained as above into an aliquot of the fluorescent antibody solution (5 ml.) at 100 mg./ml. After standing for 1 hour at room temperature with occasional stirring the supernatant is harvested by centrifugation in the cold at 18,000 r.p.m. in an angle head. The process is then repeated, or it may be replaced for the second absorption by a suitable resin (Dowex 2-chloride).

Suitable general tissues for absorption are mouse liver, pig liver and, for rabbit tissues especially, to prevent non-specific staining of eosinophils, rabbit-bone marrow.

* Both the acetone and the dioxan should be dried by distillation over sodium (metal) and by keeping over $CaSO_4$.

PREPARATION OF 1-DIMETHYLAMINONAPHTHALENE-5-SULPHONIC ACID (Mayersbach, 1957b)

Suspend 10 g. 1-aminonaphthalene-5-sulphonic acid (Laurent's acid, technical grade) in 100 ml. water in a 250 ml. stoppered flask. Add 30 ml. of 0·5 N-KOH and warm to 40° ; the Laurent's acid dissolves.

Add 10–20 ml. of dimethyl sulphate in small (2 ml.) portions, shaking the flask constantly. The addition of each portion of $(CH_3)_2SO_4$ causes the temperature to rise, and shaking should continue until it falls. A further portion of $(CH_3)_2SO_4$ is then added until the point is reached at which there is no longer a rise of temperature in the flask. On cooling, the colourless crystals of the dimethyl compound begin to separate.

Add sufficient conc. HCl to neutralize (2 ml.) ; a mass of crystals separates.

Filter through a Buchner funnel and wash with water.

Recrystallize the product from 300 ml. boiling water (twice) and dry at 80°.

Store under usual conditions. Yield 60 per cent.

PREPARATION OF 1-DIMETHYLAMINONAPHTHALENE-5-SULPHONYL CHLORIDE (Mayersbach, 1957 ; Laurence, 1957)

Place 1 g. 1-dimethylaminonaphthalene-5-sulphonic acid and 2 g. phosphorous pentachloride (PCl_5) in a wide-mouthed Pyrex tube. Mix and rub together with a stirring rod until a greyish-yellow melt is produced ; gentle heating will accelerate this process. Smear the melt over the walls of the tube. Add 50 ml. ice-cold 1·0 M-disodium phosphate and suspend the melt in this by stirring. Add 30 ml. ether and shake. The sulphonyl chloride dissolves entirely to form a dark yellow solution. Evaporate the ether under vacuum until the product crystallizes out.

Keep over $CaCl_2$, in the cold.

PREPARATION OF DANSYL CONJUGATES (Weber, 1952)

A weight of the sulphonyl chloride equal to 1–2 per cent. of the proteins is dissolved in 0·5 ml. acetone and added with stirring to 10 ml. of the protein solution in 0·1 M-phosphate buffer (pH 7·5) at 0°–3°. Leave the mixture at 0°–3° until the original turbid suspension clears (5–12 hours). Centrifuge to separate the suspended acid chloride and dialyse with stirring in the cold against 0·2 M-KCl, or 0·15 M-K_2SO_4, with frequent changes of the latter until the dialysate shows no fluorescence (6–8 days).

The combination of DANSYL with protein is a heterogeneous reaction the yield of which depends on the state of division of the acid chloride. If this is fine the originally opaque mixture becomes clear in the course of a few hours. The limiting concentration of acid chloride is 0·2 mg./ml., above which some separates as crystals and the yield decreases.

Absorption of these conjugates with liver powder, etc., is not necessary.

STAINING FOR ANTIBODY (after Coons, Leduc and Connolly)

Fresh frozen sections 2–8 μ preferably attached to slides without any adhesive, are fixed in 95 per cent. ethanol (v/v) for 15 minutes at 37°. They are then dried at 37° for 30 minutes in an upright position. For the demonstration of antibody each section is rinsed with buffered saline and covered

with a drop of a solution containing antigen in a concentration of 1/2,000 (w/v) in buffered saline. The slides are kept in a moist chamber for 30 minutes, rinsed with buffered saline and then washed in buffered saline with gentle movement for 10 minutes. They are then wiped dry except for the area of the section, covered with a drop of the absorbed antibody conjugate and again allowed to stand in a moist chamber for 30 minutes. Finally, wash as above and mount in buffered glycerol. (Pure glycerol 9 parts, buffered saline 1 part.)

Staining for Antigen

As above, omitting the intermediate antigen layering stage.

Fluorescence Photomicrography

See Chapter XXVI, p. 730, for details.

PREPARATION OF LISSAMINE RHODAMINE SULPHONYL CHLORIDE (Chadwick *et al.*, 1958)

As an alternative to DANSYL the sulphonyl chloride of the red fluorescent dye Lissamine Rhodamine B 200 (RB 200) can be employed. When conjugated with proteins this gives an orange-red fluorescence (maximum emission 610 mμ) which shows up clearly against the usual autofluorescence of the tissues.

Methods of Preparation

Grind 1 g. of RB 200 * and 2 g. PCl_5 in a mortar for 5 minutes (in a fume cupboard). Add 10 ml. *dry* acetone and stand for 5 minutes with occasional stirring. Filter and use the resulting solution for conjugation.

Method of Conjugation

Dilute each ml. of serum with 1 ml. physiological saline and 1 ml. carbonate-bicarbonate buffer (0·5 M, pH 9·0). Add 0·1 ml. RB 200 solution drop by drop with constant stirring and continue at 0°–4° with constant stirring for 12–18 hours. Dialyse against saline for 5–7 days, until the dialysate is non-fluorescent.

METHOD FOR CONCENTRATION OF PROTEIN-CONTAINING SOLUTIONS (After Kohn, 1959)

This method is based on dialysis against substances of high molecular weight.

Concentrating Procedure

Place the dilute protein-containing solution in a glass tube, or small beaker, having a protuberance or sump at the bottom end.

Break up a sufficient quantity of polyethylene glycol (Carbowax 20 M) or, alternatively, use powdered polyvinylpyrrolidone. Inflate a section of

* I.C.I. Ltd. As normally distributed the dye contains about 40 per cent. of an inert carrier. This does not affect the preparation of the sulphonyl chloride.

dialysis tubing and fill with PEG or PVP. Moisten with a small quantity of water. Insert the filled tubing into the protein solution adjusting its level to the degree of concentration required. (When the level of fluid in the glass tube falls below the bottom of the dialysis tube the concentration process ceases.)

At least 1 part of PEG should be used per 10 parts of the original solution.

APPENDIX 7

THE ALDEHYDE-FUCHSIN STAIN FOR ELASTIC TISSUE (Gomori, 1950)

(Formalin, Bouin, etc.; paraffin sections; avoid dichromate)

Preparation of the Stain

Add 1 ml. of conc. HCl and 1 ml. of paraldehyde to 100 ml. of a 0·5 per cent. basic fuchsin in 60–70 per cent. alcohol. Keep at room temperature until the shade of the mixture darkens to a deep violet (about 24 hours). The aldehyde-fuchsin stain gradually alters its properties with age, staining more rapidly and strongly when fresh.

Method

(1) Bring sections to water.
(2) Oxidize sections in Lugol's iodine for 10 minutes to 1 hour.
(3) Remove iodine with 5 per cent. thiosulphate for 1 minute.
(These two steps will remove mercury precipitate, if present.)
(4) Immerse in aldehyde-fuchsin 5 minutes to 2 hours depending on the tissue component it is desired to stain.
(5) Rinse the slide in several changes of 60–70 per cent. alcohol.
(6) Counterstain as desired—Gomori recommends hæmatoxylin-orange G, Masson's trichrome or the Mallory-Heidenhain azocarmine method, replacing aniline blue with light green or fast green in the last two cases.
(7) Dehydrate in alcohol, clear in xylene, mount in balsam or DPX.

Result

All components which take the stain appear in shades of deep purple. The optimum staining times are given in brackets. Elastic tissue (5 minutes), β-cells of the pancreas (15–30 minutes), pituitary β-granules (30 minutes to 2 hours), mast cell granules (5–10 minutes). Certain other substances and structures are stained, such as mucins and gastric chief cells.

PREPARATION OF ALDEHYDE-FUCHSIN IN DRY FORM (Rosa, 1953)

One of the main difficulties experienced in using the aldehyde-fuchsin stain is due to its instability. Especially when used for the demonstration of cells in the anterior pituitary gland, it may be found adequate for only a few days between ripening and deterioration. The dry form of the stain has overcome this difficulty. It can be made up as required and is stable for a much longer period than the dye as originally used, even for " difficult " components like pituitary β-cells.

(1) Prepare aldehyde-fuchsin according to the (original) directions given above.
(2) Allow to ripen at room temperature for 3 days.
(3) Add 100 ml. of the mixture to 50 ml. chloroform in a separating funnel and add 200 ml. distilled water.
(4) Shake briefly and allow the formed precipitate to settle.
(5) Drain off the contents of the separating funnel containing the suspended precipitate and filter *without* suction.
(6) Dry at 50° and store in a stoppered bottle.

For use add 0·5 g. to 100 ml. 70 per cent. ethanol containing 1 ml. conc. HCl.

WEIGERT'S ELASTIC STAIN (Moore, 1943)

(Various fixatives, paraffin sections)

This method depends for its success on the making of a satisfactory batch of the stain. A good batch will keep for about a year.

Preparation of the Stock Solution

Heat 500 ml. distilled water *nearly* to boiling in a large evaporating basin. Mix 2·5 g. crystal-violet (C.I. 681), 2·5 g. basic fuchsin and 1·0 g. dextrin and dissolve them in the hot water. Add 10·0 g. resorcinol and bring to the boil. When boiling add slowly 62·0 ml. of a freshly prepared 30 per cent. aqueous solution of ferric chloride (British Drug Houses, anhydrous), stirring continuously with a glass rod. The mixture must be kept boiling, but not too vigorously. Continue boiling for a further 2 minutes to coarsen the precipitate. Cool, filter through a Buchner funnel. Wash the deposit with water (8–10 litres) until no further colour is removed. Dry the precipitate overnight at 56°. Remove from the filter paper and dissolve in 550 ml. of absolute ethanol to which has been added 1 ml. conc. HCl by simmering on a water bath for 30 minutes. Cool and filter ; add 19 ml. conc. HCl and allow to stand 24–48 hours before use. The colour should be dark greenish-blue.

Preparation of the Stain

Add 35 ml. stock solution to 30 ml. 70 per cent. alcohol. If necessary these proportions can be varied.

Method

(1) Bring sections to water.

(2) Treat for 2–5 minutes with acidified permanganate (47·5 ml. of 0·5 per cent. aqueous potassium permanganate with 2·5 ml. of 3 per cent. H_2SO_4 added).

(3) Wash in water.

(4) Bleach in 1 per cent. oxalic acid for 1 minute.

(5) Wash in water and rinse in 70 per cent. alcohol.

(6) Stain in Weigert's stain for 8–24 hours (the time must depend on the results obtained since it varies considerably with each batch of the stain.

(7) Wash in 1 per cent. acid alcohol until only elastic tissue is stained.

(8) Wash in water.

(9) Counterstain lightly in carbol-safranin or neutral red.

(10) Rinse in water, dehydrate, clear and mount in a suitable synthetic resin.

Result

Elastic fibres appear dark blue-black ; nuclei, red.

VERHOEFF'S ELASTIC STAIN

(Various fixatives, paraffin sections)

Preparation of the Stain

Mix, in the order stated, 10 ml. of 5 per cent. *fresh*, unripened, alcoholic hæmatoxylin, 4 ml. of 10 per cent. *fresh* aqueous ferric chloride and 4 ml. of Lugol's iodine.

Method

(1) Bring sections down to water.
(2) Stain in Verhoeff's stain until black (15 minutes).
(3) Rinse in water.
(4) Differentiate in 2 per cent. ferric chloride until only elastic fibres and nuclei are stained.
(5) Rinse in distilled water.
(6) Counterstain with Van Gieson (10 ml. 1 per cent. acid fuchsin, 90 ml. saturated aqueous picric acid, 100 ml. distilled water, boiled for 3 minutes to ripen) for 30–60 seconds.
(7) Rinse in distilled water, dehydrate, clear and mount.

Result

Elastic fibres, black ; nuclei, grey ; collagen, red.
Background structures and cells, yellow.
The period of counterstaining must not be prolonged since the picric acid differentiates the stain further. Fine elastic fibrils are usually not stained by this method.

ORCINOL-NEW FUCHSIN METHOD FOR ELASTIC TISSUE (Fullmer and Lillie, 1956)

(*Various fixatives, paraffin sections*)

This is a modification of Weigert's technique.

Preparation of Staining Solution

Add 2 g. new fuchsin (C.I. 678) and 4 g. orcinol (highest purity) to 200 ml. distilled water and boil for 5 minutes. Add 25 ml. 30 per cent. ferric chloride (U.S.P. IX or freshly prepared from analytical reagent grade anhydrous $FeCl_3$) and boil for a further 5 minutes. Cool, collect the filtrate and dissolve this in 100 ml. 95 per cent. ethanol. In contrast to Weigert's procedure this solution is used for staining.

Method

(1) Bring sections to water.
(2) Stain for 15 minutes at 37°.
(3) Differentiate in three changes of 70 per cent. alcohol for 15 minutes in each.
(4) Dehydrate, clear and mount in synthetic resin.

Result

Elastic fibres, deep violet.
Counterstaining may be carried out with 1 per cent. aqueous safranin or with Van Gieson. The latter may be applied before or after differentiation.

SILVER IMPREGNATION FOR RETICULIN (Gordon and Sweet, 1936)

(*All ordinary fixatives, including Helly; paraffin sections*)

Method

(1) The sections should be firmly attached to the slides (egg albumin followed by heating).
(2) Bring sections to water and remove mercury deposits if necessary.

(3) Oxidize for 1–7 minutes in acidified permanganate (47·5 ml. 0·5 per cent. aqueous $KMnO_4$ with 2·5 ml. 3 per cent. H_2SO_4) for 1 minute.

(4) Wash in water.

(5) Blanch until white in 1 per cent. oxalic acid or 10 per cent. HBr for 1 minute.

(6) Wash in two changes of *glass* distilled water.

(7) Mordant for 2–15 minutes in 2 per cent. aqueous iron alum $[(NH_4)_2SO_4.Fe_2(SO_4)_3.24H_2O]$.

(8) Wash in two or three changes of distilled water.

(9) Impregnate for 5–7 seconds in Wilder's silver bath. (To 5 ml. 10 per cent. $AgNO_3$ add ammonia (28 per cent. ammonia water) drop by drop until the brown precipitate which forms is *nearly* dissolved. Add 5 ml. 3 per cent. NaOH and add ammonia drop by drop until the solution is clear. Make up to 50 ml. with glass distilled water.*)

(10) Wash briefly in distilled water.

(11) Reduce with 10 per cent. *neutral* formalin for 30 seconds.

(12) Wash in water. (If sections appear over-impregnated repeat the process from stage 7.)

(13) Tone in 0·2 per cent. yellow gold chloride for 1–3 minutes (optional).

(14) Wash in tap-water.

(15) Fix in 5 per cent. sodium thiosulphate for 5 minutes.

(16) Wash well in tap-water.

(17) Dehydrate, clear and mount.

Result

Reticulin, black ; collagen fibres, yellow to brown.

If desired a light counterstain can be employed between stages (16) and (17). The sensitivity of the method is said to be improved if stages (3)–(5) are repeated.

MALLORY'S P.T.A.H. METHOD FOR FIBRIN

(Various fixatives, paraffin sections)

Method

(1) Bring sections down to water. Remove mercury deposits with iodine and remove the iodine with alcohol, *not thiosulphate.*

(2) Postchrome for 30 minutes in a mixture of 3 parts of 3 per cent. aqueous $K_2Cr_2O_7$ and 1 part of 10 per cent. HCl.

(3) Wash in water.

(4) Differentiate for 1 minute in acid permanganate (see reticulin stain, above).

(5) Wash in water.

(6) Bleach in 1 per cent. oxalic acid until white.

(7) Rinse in water and transfer to P.T.A.H.† for 12–24 hours.

(8) Shake off excess stain (water removes the red component).

(9) Dehydrate in 99 per cent. alcohol (this differentiates the blue component).

(10) Clear and mount in a synthetic resin.

* All silver solutions must be made up in chemically clean glassware. The Wilder's silver bath will keep for 3–6 months.

† Hæmatoxylin (or hæmatein), 0·1 g. ; phosphotungstic acid, 2·0 g. ; distilled water, 100 ml. Dissolve separately and mix. Leave to ripen for several months. When ripe the stain keeps for years.

Result

Fibrin, neuroglia, red cells : dark blue.
Nuclei : light blue.
Collagen : rose red.

The balance of red and blue depends on the postchromating and removal of the chromate by the acid permanganate. The more chromate left in, the darker the blue.

BARGMANN'S CHROME HÆMATOXYLIN FOR NSS

(Bouin, Susa; paraffin sections)

NSS can be demonstrated in alcohol-fixed sections if these are floated out on Bouin's fluid instead of water.

Preparation of the Stain

1 per cent. aqueous hæmatoxylin, 50 ml.
3 per cent. aqueous chrome-alum-[$Cr_2(SO_4)_3(NH_4)_2SO_4.24H_2O$], 50 ml.
5 per cent. aqueous potassium dichromate, 2 ml.
5 per cent. aqueous H_2SO_4, 1 ml.

Allow to ripen for 48 hours before use. Keeps for several weeks at 0°–4°. Filter before use.

Method

(1) Bring sections down to water.
(2) Mordant in a solution of Bouin's fixative containing 3–4 per cent. chrome-alum for 12–24 hours at 37°.
(3) Wash in running tap-water until the sections are colourless.
(4) Oxidize for 2–3 minutes in a mixture of 2·5 per cent. $KMnO_4$ (1 part), 5 per cent. H_2SO_4 (1 part), distilled water (6–8 parts).
(5) Wash in distilled water.
(6) Bleach in 1 per cent. oxalic acid for 1 minute.
(7) Wash in running tap-water for 5 minutes.
(8) Stain for 10 minutes.
(9) Differentiate for 30 seconds in 0·5 per cent. acid alcohol.
(10) Wash in running tap-water for 2–3 minutes.
(11) Stain for 2–3 minutes in 0·5 per cent. aqueous phloxine.
(12) Rinse in 5 per cent. aqueous phosphotungstic acid (2 minutes).
(13) Wash in running tap-water for 5 minutes.
(14) Differentiate in alcohols, clear and mount.

Result (Figs. 61 and 62)

NSS : deep purple.
Nuclei : purple.
Background : pinkish-red.

HALMI METHOD FOR PITUITARY BASOPHILS (Halmi, 1952)

The original author recommends the use of Bouin's fluid, modified by substitution of 0·5 per cent. trichloroacetic acid in place of acetic acid. As reproduced below, the method works satisfactorily only in the case of the rat hypophysis.

Method

(1) Bring paraffin sections to water.
(2) Oxidize in Lugol's iodine for 30 minutes.*
(3) Remove iodine with 5 per cent. aqueous sodium thiosulphate, 2 minutes.
(4) Rinse slides thoroughly in distilled water.
(5) Stain in aldehyde-fuchsin (3–10 days old) for 2–10 minutes.
(6) Rinse in two changes of 95 per cent. alcohol and leave in 95 per cent. alcohol for 5–10 minutes.
(7) Rinse in 70 per cent. alcohol and then in distilled water.
(8) Stain in Ehrlich's hæmatoxylin (3–4 minutes).
(9) Rinse in distilled water and differentiate in 0·5 per cent. acid alcohol.
(10) Blue in running tap-water.
(11) Counterstain for 45 seconds in 0·2 per cent. aqueous light green S.F. yellowish with 1 per cent. orange G, 0·5 per cent. phosphotungstic acid, and 1 per cent. acetic acid.
(12) Rinse briefly in 0·2 per cent. acetic alcohol.
(13) Dehydrate in two changes of absolute alcohol.
(14) Blot and transfer to xylene.
(15) Mount in a suitable synthetic resin.

Result

β-granules of the rat pituitary gland stain deep purple ; δ-granules, green ; α-granules, orange.

THE PFAAB, PAS, ORANGE G METHOD FOR THE HUMAN HYPOPHYSIS (after Adams, 1956)

(*Preferred fixation is formol-mercury; paraffin sections*)

Method

(1) Bring sections to water and remove mercury deposits.
(2) Blot carefully.
(3) Apply performic acid (see p. 805) for 5 minutes.
(4) Rinse in tap-water for 10 minutes.
(5) Rinse in 70 per cent. and absolute alcohol.
(6) Blot, to flatten section on slide.
(7) Rinse in tap-water and dry section (just dry) at 60°.
(8) Rinse in absolute alcohol.
(9) Rinse in tap-water for 1 minute.
(10) Stain in acid Alcian blue (p. 806) for 1 hour.
(11) Rinse in tap-water for 5 minutes.
(12) Stain by the PAS, hæmalum, orange G sequence (p. 831).
(13) Dehydrate, clear and mount in synthetic resin.

Result (Plate IIb)

R-type mucoid granules stain magenta red.
S-type mucoid granules stain blue.
α-granules of the acidophils stain orange.
Nuclei, blue-black.

* Alternative oxidations have been employed, e.g. performic and peracetic acids, periodic acid, acid permanganate, bromine, etc. With suitable oxidation the method can be made to work on pituitary glands of other species besides the rat. Performic acid, used as on p. 806, gives good results.

THE WILSON-EZRIN METHOD FOR THE HUMAN HYPOPHYSIS
(Wilson and Ezrin, 1954)

(Formalin, formol mercury; paraffin sections)

Method

(1) Bring sections to distilled water.
(2) Oxidize in 0·5 per cent. aqueous periodic acid for 5 minutes.
(3) Treat with Schiff's reagent (p. 822) for 15 minutes.
(4) Wash in running water for 10 minutes.
(5) Stain in 1 per cent. aqueous orange G for 10–15 seconds.
(6) Mordant in 5 per cent. phosphotungstic acid for 15 seconds.
(7) Rinse in running tap-water for 15 seconds.
(8) Stain in 1 per cent. aqueous methyl blue for 1 minute.
(9) Rinse briefly in 1 per cent. acetic acid.
(10) Dehydrate in alcohols, clear and mount in a synthetic medium.

Result

The pituitary mucoid granules are divided into two types : β-granules, magenta-red ; γ-granules, purple. The α-granules are stained orange.

This method works on both rat and human hypophyses. Examination of sections by daylight with the aid of a blue filter (Ansco No. 45) brings out the contrast between red and purple.

APPENDIX 8

THE FEULGEN REACTION (Feulgen and Rossenbeck, 1924 Modified)

(*various*)

Preparation of Schiff's Reagent

Although Schiff's reagent is spoken of as if it were an analytical reagent of uniform purity and constitution it is necessary to point out that many factors in its production are not well understood and are therefore not easy to control. Firstly, not all batches of basic fuchsin will produce a satisfactory reagent and solutions made from two batches of the dye will often differ considerably in performance. Secondly, the different methods of preparation quoted in Chapter VIII do not produce a single uniform reagent. Thirdly, different conditions of use produce very different results. (See Atkinson (1952) and Longley (1952) for information on these points.)

Three methods of preparation only are given here in detail. With the first method choice of a suitable batch of basic fuchsin is especially important, and the best procedure is to obtain a number of samples of the dye and to make up a number of different solutions. When a satisfactory sample has been found a large quantity should be put into store. Thus uniform results over a long period can be attained.

Schiff's Reagent (de Tomasi, 1936). Dissolve 1 g. of basic fuchsin in 200 ml. of boiling distilled water. Shake for 5 minutes and cool to exactly 50°. Filter and add to the filtrate 20 ml. of N-HCl. Cool to 25° and add 1 g. of sodium (or potassium) metabisulphite ($Na_2S_2O_5$). Stand this solution in the dark for 14–24 hours. Add 2 g. of activated charcoal and shake for 1 minute. Filter. Keep the filtrate in the dark at 0–4°. Allow to reach room temperature before use.

Schiff's Reagent (Barger and DeLamater, 1948). Dissolve 1 g. of basic fuchsin in 400 ml. of boiling distilled water. Cool to 50° and filter. To the filtrate add 1 ml. of thionyl chloride ($SOCl_2$). Stand in the dark for 12 hours. Clear by shaking for 1 minute with 2 g. activated charcoal. Filter. Store in the dark at 0–4°. Use in the dark at room temperature.

Schiff's Reagent (Itikawa and Oguru, 1954). Boil 200 ml. distilled water and add 1 g. basic fuchsin. Shake until dissolved, cool and filter into a flask. Bubble SO_2 gas slowly through the solution through fritted glass, shaking occasionally. The SO_2 is most conveniently supplied from an SO_2 syphon.* When the solution becomes a clear transparent red colour the gas is turned off. The flask should now be stoppered and left overnight in the dark at room temperature. The pale red fluid can then be decolourized by adding 1 g. activated charcoal,* shaking for 1 minute and filtering. Store at 0–4°.

Times of Hydrolysis in N-HCl at 60°

The duration of hydrolysis varies with the fixative employed. The figures below, given in minutes, are mainly derived from K. Bauer (1932).

* British Drug Houses Ltd., Poole, Hants, England.

Apathy	5	Formalin	8
Bouin	not recommended	Formol-sublimate	8
Bouin-Allen	22	Helly	8
Bouin-Allen-sublimate	14	Newcomer (Plants)	10
Carnoy 3 : 1	6	Newcomer (Animals)	20
Carnoy 6 : 3 : 1	8	Petrunkevitch	3
Carnoy-Lebrun	6	Regaud	14
Champy	25	Regaud-sublimate	8
Chrome-acetic	14	Zenker	5
Flemming	16	Susa	18
		Zenker-formol	5

Method

(1) Bring sections to water and remove mercury if necessary.
(2) Rinse briefly in cold N-HCl.
(3) Place in N-HCl at 60° for the optimum time of hydrolysis.
(4) Rinse briefly in cold N-HCl and then in distilled water.
(5) Transfer to Schiff's solution for the optimum time ($\frac{1}{2}$–1 hour with de Tomasi, longer with Barger and DeLamater).
(6) Drain and rinse in three changes of freshly prepared bisulphite solution (5 ml. 10 per cent. $K_2S_2O_5$, 5 ml. N-HCl, water to 100 ml.).
(7) Rinse in water.
(8) Counterstain if desired (1 per cent. aqueous light green, 1 minute, or 0·5 per cent. alcoholic fast green, $\frac{1}{2}$–1 minute).
(9) Dehydrate in alcohol.
(10) Clear in xylene and mount in balsam or DPX.

Result (Fig. 64, p. 203)

DNA appears in shades of reddish-purple.

THE FEULGEN-NAPHTHOIC ACID HYDRAZIDE REACTION (Pearse, 1951)

(*various*)

Preparation of 2-hydroxy-3-naphthoic Acid Hydrazide (NAH) (Seligman, Friedman and Herz, 1949)

(1) Prepare the acid chloride by warming 20 g. of 2-hydroxy-3-naphthoic acid with 25 ml. of thionyl chloride until the solid has dissolved and HCl is no longer evolved.
(2) Remove excess thionyl chloride by distillation at reduced pressure. The acid chloride solidifies on cooling and is dissolved in 50 ml. of methanol (pure).
(3) Remove the methanol by distillation at low pressure.
(4) Add 35 ml. of 85 per cent. hydrazine hydrate and heat on a steam bath for 3 hours. The hydrazide separates as a crystalline mass on cooling.
(5) Add water and collect the crystals with suction. Wash in water.
(6) Dissolve crystals in 500 ml. of hot ethanol and treat with activated charcoal.
(7) Chill the solution, collect the crystals and wash with ether.
Yield. 16 g. (75 per cent.). Pale yellow platelets, M.P. 203°–204°.

Method

(1) Bring sections to water.
(2) Rinse briefly in cold N-HCl.
(3) Place in N-HCl at 60° for the optimum time of hydrolysis (as for Feulgen reaction).
(4) Rinse briefly in cold N-HCl, in distilled water, and finally in 50 per cent. alcohol.
(5) Treat sections at about 22° for 3–6 hours with 0·1 per cent. NAH in 50 per cent. ethanol with 5 per cent. acetic acid.
(6) Rinse in three changes of 50 per cent. alcohol, 10 minutes in each change.
(7) Rinse in water.
(8) Transfer to a freshly prepared solution of diazotized *o*-dianisidine,* at 0° and pH 7·4, for 1–3 minutes.
(9) Wash in water.
(10) Dehydrate in alcohol, clear in xylene and mount in DPX.

Result (Fig. 65)

DNA bluish-purple. Cytoplasmic and other proteins, especially if strongly basic, may be stained pinkish red.

MODIFIED TURCHINI METHOD FOR DNA AND RNA (after Blackler and Alexander, 1952)

(*Formal-mercury, Zenker, Bouin, paraffin sections*)

Preparation of the Reagent. (9-methyl-2,3,7-trihydroxy fluorone)

Mix 1 mole of 1,2,4-triacetyl-trioxybenzene, 1·25 moles of paraldehyde in a quantity of reagent ethanol 5 times the weight of the two reactants. Add 5–10 per cent. (v/v) sulphuric acid. Allow to stand for 18–24 hours at room temperature. Add 30 volumes of distilled water. Stand for 24 hours. A reddish-orange precipitate of the dye settles out. Filter and discard the filtrate. Dry the product at 37–40°. Redissolve the dye in the minimum volume of ethanol and filter. Add 30–60 volumes of water to the filtrate and stand for 24 hours. Filter and dry as above. The reddish-orange powder decomposes at 319°. It is moderately soluble in alcohol and practically insoluble in water.

Method

(1) Bring sections to water as usual.
(2) Hydrolyse in N-HCl for 6–12 minutes at 60°.
(3) Transfer to 80 per cent. ethanol for 15 seconds.
(4) Immerse in the fluorone solution for 4–14 hours. (Dissolve 0·5 g. of the dye† in 100 ml. 95 per cent. ethanol containing 1 ml. H_2SO_4 ; filter before use).
(5) Transfer to 1 per cent. aqueous Na_2CO_3 for 2 minutes.
(6) Immerse in distilled water for 2 minutes.
(7) Dehydrate in 50 per cent. acetone, and in 100 per cent. acetone.
(8) Clear in acetone-xylene and in xylene.
(9) Mount in a suitable synthetic resin.

* The stable diazotate, Fast Blue B salt (I.C.I. Ltd.) may be used as an alternative.
† Obtained from L. Light & Co., Colnbrook, Bucks, England.

Result

DNA in chromatin bluish-purple ; RNA reddish-orange.

If alcoholic differentiation is used some of the dye is extracted from the nucleic acids.

THE METHYL-GREEN-PYRONIN AND RIBONUCLEASE METHOD FOR RNA (Brachet, 1942)

(*various*)

Short fixation in 10 per cent. formalin, at pH 7·0 ± 0·2 for 4–16 hours, is recommended.

The use of ribonuclease is considered in Chapter XXII, and of the various solutions for the extraction of RNA (and DNA) in Chapter VIII. Any of these methods can be used in conjunction with methyl green-pyronin staining.

Preparation of Methyl Green Solutions

Samples of methyl green are always mixtures of this dye and methyl violet, from which the former dye differs only in possessing 7 instead of 6 methyl groups. Before use as a histochemical agent methyl green should always be freed from the violet component by shaking an aqueous solution with excess of chloroform or amyl alcohol, both of which dissolve methyl violet. Preferably after two or three days' standing the aqueous supernatant is removed for use. Methyl green purified in this way tends to break down slowly, by loss of the seventh methyl group, into methyl violet. The rate of conversion over a period of up to 5 years is nearly negligible.

Preparation of Methyl Green-pyronin Solutions

Pyronin G or pyronin Y are usually recommended but many samples of these dyes are not suitable for the present purpose. On the whole the bluish shades perform most satisfactorily but it is often necessary to test a number of samples.

The older methyl green-pyronin solutions were made up in a dilute alcoholic solution containing 0·5 per cent. phenol. These are not satisfactory in practice and the method given below, modified from Trevan and Sharrock (1951), can be substituted. Alternatively, and especially if spectrophotometric measurements are intended, the method of Kurnick, also given below, is recommended.

Solution A. Five per cent. aqueous pyronin 17·5 ml., 2 per cent. aqueous methyl green (chloroform-washed) 10 ml., distilled water 250 ml.

Solution B. M/5 acetate buffer, pH 4·8. In the original directions this solution contains 30 ml. of 1 per cent. orange G, but for purely histochemical work this should be omitted.

For use mix equal volumes of A and B in a Coplin jar. The mixture keeps for about a week, it should not be used for longer.

Method

(1) Bring sections to water.
(2) Stain in methyl green-pyronin solution 10 minutes to 24 hours.
(3) Rinse in distilled water for a few seconds. (Some pyronin is removed at this stage, which must be kept short.)
(4) Blot dry.
(5) Dehydrate rapidly in absolute acetone.
(6) Rinse briefly in equal parts of acetone and xylene.

(7) Rinse briefly in 10 per cent. acetone in xylene.
(8) Clear in two changes of clean xylene.
(9) Mount in DPX.

Result

Nuclear chromatin : green, bluish-green or purplish-green.
RNA : red.

The purplish-red staining of the nuclei, so often obtained with the older methods, was partly due to the admixture of methyl violet and partly to the low pH of the staining solutions. At low pH levels the pyronin component stains more strongly and at higher levels, from pH 4·5 to 5·5, the effect of methyl green is accentuated. Before extraction of RNA the majority of nuclei contain some pyronin ; after extraction with ribonuclease the nuclei are green but with other methods, due to depolymerization of DNA, they may take up even more pyronin than before extraction.

METHYL-GREEN-PYRONIN Y METHOD FOR DNA AND RNA (Kurnick, 1955)

(*Carnoy, Wolman 22° ; cold microtome or paraffin sections*)

Preparation of Stain

Make up a 2 per cent. aqueous solution of Pyronin Y.* Extract with $CHCl_3$ by shaking in a separating funnel until the chloroform layer becomes colourless. Make up a 2 per cent. aqueous solution of methyl green and extract with $CHCl_3$, as above, until the chloroform layer is no longer violet coloured. For use mix 12·5 ml. Pyronin Y solution and 7·5 ml. of methyl green with 30 ml. distilled water.

Method

(1) Bring paraffin sections to water ; cold microtome sections can be immersed directly in the staining mixture.
(2) Stain for 6 minutes in methyl green-pyronin.
(3) Blot with filter paper.
(4) Immerse in 2 changes of *n*-butyl alcohol,† 5 minutes in each.
(5) Immerse in xylene for 5 minutes.
(6) Immerse in cedar oil for 5 minutes.
(7) Mount in Permount.

Result (Figs. 66–69)

Chromatin clear green, nucleoli bright red, cytoplasmic RNA bright red. Eosinophil granules and osteoid also stain bright red.

THE GALLOCYANIN-CHROMALUM METHOD FOR NUCLEIC ACIDS (Einarson, 1951)

(*various*)

Preparation of the Staining Solution

Dissolve 5 g. of chromalum ($K_2SO_4.Cr_2(SO_4)_3.24H_2O$) in 100 ml. of distilled water. Add 0·15 g. gallocyanin and mix by shaking. Warm

* Kurnick found only Pyronin Y (05564, G. T. Gurr) or Pyronin Y (Edward Gurr) suitable for the purpose. He considers that Pyronin B, which differs from Pyronin Y in being tetraethyl instead of tetramethyl compound, gives only non-specific counter-staining of cytoplasmic components.

† Tertiary butyl alcohol cannot be used for differentation.

gradually and bring to the boil. Boil for 5 minutes. Cool to room temperature, filter and add distilled water through the filter paper until the volume of the filtrate reaches 100 ml. The pH of this stock solution, which is ready for use, is 1·64 (lasts 4 weeks).

The pH of the staining solution may be altered by adding given amounts of 1·0 M HCl and 1·0 M NaOH, as indicated in the Table below, to 40 ml. of the stock solution,

ml. 1·0 M HCl	pH	ml. 1·0 M NaOH	pH
10	0·83	0	1·64
9	0·90	1	1·84
8	0·92	2	2·16
7	0·94	3	2·90
6	1·02	4	3·42
5	1·10	5	3·76
4	1·14	6	3·98
3	1·18	7	4·07
2	1·29	8	4·18
1	1·44	9	4·27
0	1·64	10	4·35

The addition of strong acid or base to the stock solution does not cause any abrupt change in pH. After the addition of NaOH a precipitate of dye-lake-hydroxide occurs and the solution should not be used for longer than 7 days.

Method

(1) Bring sections to water.
(2) Stain in gallocyanin-chromalum for 48 hours at room temperature.
(3) Wash briefly in water.
(4) Dehydrate in the alcohols.
(5) Clear in xylene and mount in DPX.

Result

Nucleic acids stain deep blue. Depending on the pH of the staining solution, other structures may be stained. At pH 1·64 cartilage gives a fine red metachromasia. At low pH values (0·83 to 0·94) staining of structures other than the nucleic acids is very slight. From pH 1·1 to 2·9 this non-specific staining increases and it reaches a maximum between pH 3·3 and 3·5, falling abruptly at pH 4·07. Staining of the nucleic acids does not vary in this way.

MODIFIED GALLOCYANIN METHOD FOR NUCLEIC ACIDS (de Boer and Sarnaker, 1956)

(*Formalin, frozen sections ; various fixatives, paraffin sections*)

Preparation of the Dye Solution

Shake up 600 mg. of gallocyanin in 200 ml. disilled water for one minute. Filter and discard the filtrate. Transfer the filter paper and residue to 200 ml. of 5 per cent. chrome alum solution in distilled water. Place on a water bath

and boil for 30 minutes. Cool, filter and adjust filtrate to pH 1·6 with 1 per cent. HCl

Method

(1) Bring sections to water.
(2) Stain in gallocyanin-chromalum for 24 hours or longer (depending on the age of the stain).
(3) Rinse for 1 minute in distilled water at pH 1·6. (Acidified with HCl).
(4) Repeat rinse until no further dye can be removed.
(5) Dehydrate in alcohol, clear and mount in DPX.

Result

Nucleic acids stain deep blue. Nissl granules are especially well shown. The non-specific staining of the original method is substantially reduced by the procedures outlined above.

THE EOSIN-GRAM-WEIGERT METHOD

(*Formalin and various other fixatives ; paraffin sections*)

A large number of variations of this method exist. If it is proposed to employ the method, in comparative studies, as an index of the physical structure of protein components of the tissues, it is essential to standardize every detail of the technique.

Method

(1) Fix tissues in 10 per cent. formol saline for 4 days.
(2) Wash for 12–24 hours in running water.
(3) Embed in paraffin by any standard technique. (The period in hot wax should be constant in each case.)
(4) Cut sections 5–6 μ, float on water, mount on egg-albuminized slides.
(5) Dry, preferably in paraffin-oven at 60°, for a constant time ($\frac{1}{2}$–1 hour).
(6) Remove wax by dipping in light petroleum (avoid hot stage).
(7) Transfer to absolute acetone, absolute alcohol, 70 per cent. alcohol and finally to water.
(8) Stain in 1 per cent. aqueous Eosin for 10–15 seconds.
(9) Wash briefly in water and blot dry.
(10) Stain in freshly filtered aniline-Gentian violet, 2 minutes. (Saturated alcoholic crystal violet, 16 ml., 2 per cent. aniline water to 100 ml.)
(11) Wash briefly and flood with Gram's iodine, 2 minutes. (Iodine 1 g., KI 2 g., distilled water 300 ml.)
(12) Wash briefly and blot well.
(13) Differentiate, with frequent blotting, in aniline-xylene (equal parts). A suitable time must be arrived at by experiment (say that necessary to give positive staining in normal collagen), and this time must be used as a constant for any one series of slides.
(14) Blot.
(15) Wash in 2 changes of xylene.
(16) Mount in DPX.

Result (Fig. 75)

Gram-positive structures stain purple in varying degrees.

ALKALINE FAST GREEN FOR BASIC PROTEINS OF NUCLEI (Alfert and Geschwind, 1953)

(10 *per cent. neutral formalin, paraffin sections*)

Method

(1) Fix tissues for 3–6 hours in 10 per cent. neutral formalin, wash overnight in running water, dehydrate, clear and embed in paraffin.

(2) Bring sections to water.

(3) Immerse for 15 minutes in 5 per cent. aqueous trichloroacetic acid at 100°.

(4) Wash in 3 changes of 70 per cent. alcohol, 10 minutes in each.

(5) Wash in distilled water.

(6) Stain at 22° for 30 minutes in 0·1 per cent. aqueous fast green F.C.F. adjusted to pH 8·0 to 8·1 with the minimum amount of NaOH.

(7) Wash in distilled water for 5 minutes.

(8) Dehydrate in 95 per cent. alcohol, clear and mount in a suitable synthetic resin.

Result (Fig. 73).

Basic proteins stain bright green, due to their content of arginine and lysine.

NAPHTHOL-YELLOW S METHOD FOR BASIC PROTEINS OF NUCLEI (Deitch, 1955)

(*Freeze-dried, alcohol post-fixed; cold microtome, acetic-ethanol-fixed; Formalin, Carnoy etc., paraffin sections*)

Method

(1) Bring sections to water.

(2) Stain for 4–6 hours in 0·5 per cent. aqueous Naphthol Yellow S at pH 2·7.

(3) Rinse in distilled water.

(4) Dehydrate in 95 per cent. alcohol, clear and mount.

Result

The amount of yellow staining ($\lambda^{max} = 435$ mμ) reflects the number of available basic groups of the protein.

In the case of the nuclei, if the nucleic acid is not previously removed, only those basic groups not blocked by DNA will be demonstrated.

EXTRACTIVE METHODS FOR NUCLEIC ACIDS

(Methods using enzymes are given in Appendix 22)

Perchloric Acid (after Erickson *et al.*, 1949)

(*formalin, formol sublimate; paraffin sections*)

For removal of RNA alone, bring sections to water, after removal of mercury where necessary, and treat with 10 per cent. perchloric acid at 4° for 12–18 hours.

For removal of both nucleic acids treat with 5 per cent. perchloric acid at 60° for 20–30 minutes.

Neutralize sections in 1 per cent. sodium carbonate (1–5 minutes), wash, and stain with 1 per cent. aqueous toluidine blue.

Bile Salts (after Foster and Wilson, 1952)

(*preferably formalin-fixed paraffin sections*)

Bring sections to water and treat for 24–48 hours at 60° in a 2 per cent. aqueous solution of sodium tauroglycocholate. It is essential that this extractive solution be kept continuously oxygenated by means of a stream of oxygen. After incubation, wash the sections in water and stain with toluidine blue or by Gram's method. Only RNA is released by bile salt extraction.

Trichloroacetic Acid (after Schneider, 1945)

(*various, paraffin sections*)

Bring sections to water and treat with 4 per cent. trichloroacetic acid at exactly 90 ° for 15 minutes. Wash and stain with toluidine blue or in White's (1950) orange G-aniline blue mixture. Both types of nucleic acid are extracted by this procedure.

Hydrochloric Acid (after Dempsey *et al.*, 1950)

(*Zenker etc., paraffin sections*)

Bring sections to water and treat with 1 N-HCl for 3 hours at 37°. Wash, and stain with M/2000 methylane blue at pH 5·7 for 12–24 hours, or with any other suitable basic dye for shorter periods. This method removes both types of nucleic acid.

APPENDIX 9

THE PERIODIC ACID-SCHIFF TECHNIQUE (after Hotchkiss, 1948)

(most types of fixation ; frozen or paraffin sections)

Preparation of the Solutions

(1) **Periodic Acid.** Dissolve 0·4 g. of periodic acid (HIO_4, $2H_2O$) in. 35 ml. of reagent ethyl alcohol and add 5 ml. of M/5 sodium acetate (27·2 g of the hydrated salt in 1,000 ml.) and 10 ml. of distilled water. This solution should be kept in the dark at 17°–22° and used at this temperature. It should be discarded if a brown colour appears.

(2) **Reducing Bath.** Dissolve 1 g. potassium iodide and 1 g. sodium thiosulphate ($Na_2S_2O_3$, $5H_2O$) in 30 ml. of reagent ethyl alcohol and 20 ml. of distilled water. Add 0·5 ml. of 2 N-HCl (20 per cent. conc. HCl). A deposit of sulphur forms which can be ignored. Keep between 17°–22° ; the solution lasts for about 14 days, not longer.

(3) **Schiff's Reagent.** For the preparation of this reagent see Appendix 8, p. 822. For most purposes the reagent of Barger and DeLamater is recommended.

(4) **Celestin Blue Solution.** Dissolve 2·5 g. iron alum in 50 ml. distilled water by standing overnight at room temperature. Add 0·25 g. Celestin blue R and boil for 3 minutes. Filter when cool and add 7 ml. glycerol.

(5) **Orange G Solution.** Dissolve 2 g. orange G (C.I. No. 27) in 100 ml. of 5 per cent. aqueous phosphotungstic acid. Stand for 24 hours and use the supernatant.

Method (short title PARS)

(1) Bring sections down to water and remove mercury.
(2) Rinse in 70 per cent. alcohol.
(3) Immerse in periodic acid solution for 5 minutes.
(4) Rinse in 70 per cent. alcohol.
(5) Immerse in the reducing bath for 1 minute.
(6) Rinse in 70 per cent. alcohol.
(7) Immerse in Schiff's solution (Barger and DeLamater) 20 minutes.
(8) Wash in running water for 10 minutes.
(9) Stain nuclei lightly with celestin blue 2–3 minutes, followed by Mayer's hæmalum 2–3 minutes.
(10) Differentiate in 1 per cent. acid alcohol.
(11) Wash in running water for 30 minutes.
(12) Counterstain with orange G for about 10 seconds.
(13) Wash in water till sections are pale yellow (about 30 seconds).
(14) Dehydrate in alcohol, clear in xylene, mount in DPX.

Stage 14 may be completed after stage 8, or after stage 11, as well as in its final position. The resulting variations (PAS, Dipas, Tripas) will be found useful for different purposes. Tripas or Trichrome-PAS (Pearse, 1949, 1950b) has been used as a routine stain for the human and many animal hypophyses. Dipas is useful in cases where orange G overstaining of PAS-stained components is not desired. Alternatively, the PAS stain

may be followed by orange G, omitting the nuclear stain altogether. This variant is useful for differential cell counts in the animal hypophysis. For all other purposes the method given below gives more satisfactory results.

Result (Plate IB)

Mucoproteins and neutral mucopolysaccharides, deep purplish-red. Glycoproteins are usually paler red or pink. (This list is extremely abbreviated.) In the full method (Trichrome-PAS) the nuclei are blue-black and RBC's and acidophil proteins are yellow.

THE PERIODIC ACID-SCHIFF TECHNIQUE (after McManus)

(*various*)

Method (short title PAS)

(1) Bring sections to water and remove mercury, if necessary.*

(2) Oxidize for 2–5 minutes, not longer, in 0·5 per cent. aqueous periodic acid.

(3) Wash in distilled water.

(4) Immerse in Schiff's reagent 10–15 minutes.

(5) Wash in running water for 5–10 minutes.

(6) Counterstain nuclei with the celestin blue-hæmalum sequence (Stage 9 of the previous method).

(7) Differentiate if necessary in 1 per cent. acid alcohol and follow by thorough washing in running water.

(8) Dehydrate in alcohol, clear in xylene and mount in a suitable synthetic medium.

Result

Carbohydrate-containing proteins stain in various shades of purplish-red. Glycogen stains deeply.

PREVENTION OF PAS REACTION BY ACETYLATION

(Lillie, 1954a)

Control sections are treated at 22° for 1–24 hours in 16 ml. acetic anhydride in 24 ml. of dry pyridine, washed in water and then subjected to the PAS reaction as above. A negative reaction after acetylation indicates that 1 : 2-glycol groups were responsible for the original reaction observed. The following double procedure follows that recommended by McManus and Cason (1950).

(1) Bring three sections to water.

(2) Leave one section (A) untreated until stage 7.

(3) Treat the second and third sections (B and C) in the acetic anhydride-pyridine mixture.

(4) Wash sections B and C in water.

(5) Treat section C with 0·1 N-KOH for 45 minutes at room temperature

* When using fresh or formalin-fixed frozen sections it has been customary to block existing aldehydes by one of the techniques for this purpose given in Appendix 12, pp. 865 and 866. Recently Lillie (1956) has shown that cyanide blockade of aldehydes is reversed by oxidation with HIO_4 which apparently is able to attack cyanohydrins successfully. Staple (1958) has shown that other forms of aldehyde blockade are reversible in this way but, according to Lillie, aniline and phenylhydrazine block are not so reversible.

or with 20 per cent. ammonia in 70 per cent. alcohol at 37° (Lillie) for 24 hours.

(6) Wash this section in water.

(7) Treat all three sections by the PAS routine.

A positive result in a given structure in sections A and C with a negative result in section B confirms the carbohydrate nature of the reacting groups.

THE LEAD TETRAACETATE-SCHIFF METHOD

(Shimizu and Kumanoto, 1952)

(paraffin sections after various fixatives)

Preparation of Solution

Dissolve 1 g. $Pb(O.CO.CH_3)_4$ in 30 ml. glacial acetic acid. Immediately before use add 70 ml. saturated sodium acetate solution.

Method

(1) Bring sections to water.
(2) Immerse in 1·0 M-sodium acetate, 5 minutes.
(3) Treat with fresh tetraacetate solution, 10 minutes.
(4) Immerse in 1·0 M-sodium acetate, 5 minutes.
(5) Wash in running water, 10 minutes.
(6) Treat with Schiff's reagent, 15 minutes.
(7) Treat with sulphite water (as in Feulgen reaction, Appendix 8, p. 823).
(8) Wash in running water, 10 minutes.
(9) Dehydrate, clear and mount in Canada balsam.

Result

Glycogen and various mucins and mucoproteins are stained reddish-purple by this method, as with the PAS methods.

SODIUM BISMUTHATE-SCHIFF METHOD

(after Lhotka)

(paraffin sections after most fixatives)

Method

(1) Bring sections to water.

(2) Oxidize for 3 minutes in 1 per cent. sodium bismuthate in 20 per cent. orthophosphoric acid (mix 40 ml. 1·25 per cent. bismuthate with 10 ml. of acid and use immediately).

(3) Wash in running tap-water for 1 minute.

(4) Rinse for 15 seconds in N-HCl to remove adherent bismuth pentoxide and rinse in distilled water.

(5) Immerse in Schiff's reagent for 10 minutes.

(6) Treat with sulphite water (as in Feulgen reaction, Appendix 8, p. 823).

(7) Wash in running water.

(8) Dehydrate, clear and mount in a suitable resin.

Result

Staining is similar to that obtained with the PAS and $PbAc_4$-S methods.

METACHROMATIC METHODS

(fresh frozen or formalin-fixed frozen ; various paraffin)

Schmorl's Thionin Method

Method

(1) Bring paraffin or frozen sections to water.
(2) Treat with saturated aqueous mercuric chloride, 30 seconds.
(3) Wash quickly in water.
(4) Stain in dilute aqueous thionin (2 drops of a hot-saturated solution in 5 ml. of distilled water) for 5–15 minutes.
(5) Wash briefly in distilled water.
(6) Mount frozen and paraffin sections preferably in glycerine jelly.

Result

Metachromatic substances red, nuclei blue.

Toluidine Blue Method (Standard)

Different samples of this dye behave differently in respect of metachromasia and particularly in respect of the resistance of this metachromasia to the usual process of dehydration in alcohol. Selection of a suitable sample of the dye is, therefore, of paramount importance.

Method

(1) Bring sections to water.
(2) Stain in 0·5 per cent. aqueous toluidine blue for 4–6 hours.
(3) Rinse in distilled water.
(4) For histochemical purposes examine immediately in water.
(5) Mount in glycerine jelly.

Result (Plate Ia)

Metachromatic substances red or pink (γ-metachromasia) or purple (β-metachromasia). Nuclei blue (usually). An orange-yellow filter alters the shift from blue to reddish-purple to one from green to red.

Toluidine Blue Method (Kramer and Windrum)

(fixation unimportant)

Method

(1) Bring sections to water.
(2) Stain in 0·1 per cent. toluidine blue in 30 per cent. ethanol for 5–20 minutes.
(3) Rinse in 95 per cent. alcohol.
(4) Dehydrate in absolute alcohol.
(5) Clear in xylene and mount in a suitable synthetic resin.

Result

γ-metachromasia red or pink ; β-metachromasia purple.

Toluidine Blue Method for Permanent Preparations (after Hess and Hollander, 1947)

(Zenker-fixed paraffin sections)

Method

(1) Bring sections to water.
(2) Cover for 30 seconds with toluidine blue solution (0·25 g. toluidine blue in 100 ml. 0·25 per cent. borax in distilled water).
(3) Rinse with 0·25 per cent. borax solution.
(4) Apply a solution of 0·5 per cent. potassium dichromate, previously saturated with mercuric chloride and filtered, for 15 seconds.
(5) Add fresh dichromate-sublimate solution and leave for 2 minutes.
(6) Blot dry.
(7) Immerse in absolute alcohol for 10 seconds with constant agitation.
(8) Immerse in clean xylene for 30 seconds.
(9) Rinse in absolute alcohol and drain.
(10) Cover with the aqueous dichromate-sublimate solution for 30 seconds.
(11) Rinse in colophony-alcohol (1 g. colophonium resin, 100 ml. alcohol) and drain.
(12) Clear in two changes of benzene.
(13) Mount in Canada balsam dissolved in benzene.

Result

The red (γ) metachromasia is preserved and in some cases even enhanced by the treatment with resin-alcohol.

SULPHATION METHODS FOR INDUCING METACHROMASIA (after Kramer and Windrum, 1954)

Methods

(1) Conc. H_2SO_4. Treat sections for 60–75 seconds. Remove and wash in running tap water. Stain.*
(2) Chlorosulphonic acid in pyridine. Care must be taken in the preparation of this reagent and the pyridine must be dry. Add 11 ml. chlorosulphonic acid to 100 ml. pyridine contained in a flask immersed in an ice-water-salt mixture. The reaction product is solid at room temperature and is used at 65–70° for 5 minutes. Sections are then washed in water and stained.*
(3) Sulphuric-acetic anhydride. Equal volumes of sulphuric acid and acetic anhydride mixed slowly in a cooled vessel. Immerse sections for 3 minutes, then wash and stain.*
(4) Chlorosulphonic-acetic-chloroform. Mix 5 ml. chlorosulphonic acid, 10 ml. acetic acid and 12 ml. chloroform. Use immediately. Immerse sections for 5 minutes at 22°, wash and stain.*
(5) Fuming sulphuric-acetic anhydride-ether. Mix 35 ml. acetic anhydride and 15 ml. ether. Add 50 ml. of fuming H_2SO_4, drop by drop. Immerse sections for 2–3 minutes only. Wash and stain.*
(6) Sulphuric ether (Gomori). Add 80 ml. fuming H_2SO_4 carefully to 20 ml. dry ether. Immerse sections for 2–6 minutes. Wash and stain.*

* 0·01 per cent. Azure A (Hopkins and Williams Ltd.) in 30 per cent. ethanol.

METHYLENE BLUE EXTINCTION (after Dempsey and Singer, 1946)

(various fixatives, paraffin sections)

Preparation of Solutions

Because of the differing extractive tendencies, and the different effects on staining affinities, of the various buffer solutions it is preferable to prepare the whole range of methylene blue solutions with a single buffer. For this purpose the veronal-acetate buffer of Michaelis (1931) is advised. Details are given in Appendix 1, p. 781. A series extending from pH 8·18 to pH 2·62 is adequate for most investigations.

In each buffer solution dissolve methylene blue to give a concentration of 0·0005 M. Place the solutions in stoppered tubes rather than in Coplin jars.

Method

(1) Bring sections to water, removing mercury precipitate if necessary.

(2) Immerse sections in the buffered methylene blue solutions for 24 hours at room temperature (17°–22°, avoid great variation).

(3) Wash quickly in distilled water and examine in water.

(4) Mount in glycerine jelly.

Result

The intensity of staining varies directly with the pH. Colorimetric measurements were made by the original authors to compare the binding capacity for methylene blue of various basophilic substances over a wide range of pH.

The point at which virtual extinction of staining occurs is taken as the methylene blue extinction of the tissue component concerned.

DIALYSED IRON METHOD FOR ACID MUCOPOLYSACCHARIDES (Hale, 1946)

(Unfixed fresh frozen sections or fresh frozen sections briefly fixed in Wolman's fixative are excellent. Carnoy or formalin-fixed paraffin sections usually recommended)

Method

Sections should be mounted on the slides without any adhesive.

(1) Bring sections to water, removing mercury precipitate where necessary.

(2) Flood with dialysed iron (1 vol. dialysed iron, B.D.H.,* 1 vol. 2 M-acetic acid) for 10 minutes.†

(3) Wash well with distilled water.

(4) Flood with acid ferrocyanide solution (0·02 M-$K_4Fe(CN)_6$, 0·14 M-HCl, equal parts), leave for 10 minutes.

(5) Wash in water.

(6) Counterstain nuclei with Mayer's carmalum (6–18 hours) or with 1 per cent. aqueous neutral red (1 minute). Counterstaining is not recommended in the case of frozen sections.

* British Drug Houses Ltd., Poole, England.

† The colloidal iron reagent of Rinehart and Abu'l Haj (1951) is made by dissolving 75 g. of $FeCl_3$ in 250 ml. distilled water, adding 100 ml. glycerol and then, gradually, 55 ml. 28 per cent. ammonia. This last should be added with constant stirring. The mixture is dialysed against distilled water for three days with regular changes of the water. The staining time with this mixture is also about 10 minutes.

(7) Dehydrate in alcohol, clear in xylene and mount in DPX or any suitable synthetic resin.

Result (Figs. 77 and 84)

Strongly acidic polysaccharides are stained blue, nuclei red.

COMBINED DIALYSED IRON AND PAS STAIN FOR POLYSACCHARIDES (after Ritter and Oleson, 1950)

(paraffin sections after formol-alcohol, formalin or Helly)

This method was designed by the authors to demonstrate two separate classes of carbohydrate-containing proteins. Its use for this purpose, as a purely histochemical procedure, is not permissible. Nevertheless, it has important applications to histology. As shown by McManus, Lupton and Graham (1951), the method shows the intereapillary space in human glomeruli particularly well.

Method

Fix tissues (as fresh as possible) in 10 per cent. formalin in 90 per cent. alcohol. The other fixatives mentioned above are adequate. Embed in paraffin wax.

(1) Bring sections to water.
(2) Perform Hale's method, stages 2–5 inclusive.
(3) Wash in water.
(4) Apply the PAS reaction, stages 2–5 inclusive.
(5) Counterstain nuclei if desired, lightly, with Mayer's hæmalum, 4–6 minutes.
(6) Wash well in water.
(7) Dehydrate in alcohol, clear in xylene, mount in Canada balsam or DPX.

Result

Acid mucopolysaccharides, blue. Most protein struetures, pale blue. Nuclei, pale blue or dark blue if counterstained. Mucoproteins, purplish red.

In the kidney the visceral glomerular basement membrane is blue, the parietal glomerular, and the tubular, basement membranes are red. The glomerular intercapillary space is red.

THE BI-COL METHOD (Wolman, 1956)

(Carnoy-fixed paraffin sections)

Preparation of Solutions

(1) *Colloidal iron* according to the method of Rinehart and Abu'l Haj, given above.

(2) *Colloidal gold.* Add 2·5 ml. of a 0·2 per cent. gold chloride solution (H Au Cl_4) to 90 ml. of doubly-distilled water. Prepare a 10 per cent. alkaline formalin by diluting 40 per cent. formalin (reagent grade) 1 : 10 with 0·5 per cent. KOH. Add 2 ml. of this solution to the gold chloride solution with vigorous stirring. Boil the resulting solution for 15–30 minutes. The final solution should be red and of pH 5 or thereabouts. It should be used on the day of preparation.

Method

(1) Bring sections to distilled water.
(2) Immerse in the colloidal iron reagent for 10 minutes.
(3) Develop with acid ferrocyanide as in Hale's method after washing in distilled water.
(4) Treat with colloidal gold for 18–24 hours at 37°.
(5) Rinse in tap-water.
(6) Dehydrate, clear and mount in Canada balsam.

Result

Chromatin (nuclei) blue or brownish-red. Compounds containing free phosphoric or sulphuric radicals stain blue. Those containing weak acids stain reddish-brown. Hyaluronic and stained blue *in vitro* but, according to Wolman, reddish-brown in the tissues. Sulphated hyaluronic acid would of course stain blue.

THE ALCIAN BLUE METHOD FOR ACID MUCOPOLYSACCHARIDES (after Steedman, 1950)

(*suitable for all types of material, fixed and unfixed ; for paraffin sections fixation in Bouin, Carnoy, formalin or formol-mercury is recommended*)

Method

(1) Bring sections to water removing mercury precipitate where necessary.
(2) Stain in a freshly filtered 0·1 per cent. solution of Alcian Blue in 3 per cent. acetic acid for 10–30 minutes.
(3) Rinse in distilled water.
(4) Stain in Ehrlich's hæmalum 5–10 minutes, or in 1 per cent. Neutral red,* 30 seconds, or in 0·5 per cent. Chlorantine fast red 5B (Lison, 1954b) 10–15 minutes.
(5) Differentiate in 1 per cent. alcohol. } Omit if using Neutral red or
(6) Wash in running water 10–20 minutes. } Chlorantine fast red.
(7) Dehydrate in alcohol, clear in xylene and mount in Canada balsam or in DPX.

If the use of stains in acid solution is to follow staining with Alcian Blue, the following manœuvre may be performed after stage 3 : Immerse in 1 per cent. alkaline alcohol (pH 8 or over) for 2 hours or more to convert the dye into the insoluble pigment Monastral Fast Blue.

Result (Plate IIc)

Acid mucopolysaccharides, clear blue-green. Nuclei, dark blue or dark red. In place of Alcian blue 8GS, Alcian green 3BX or Alcian green 2GX can be employed.

* If connective tissue mucins (hyaluronic acid, chondroitin sulphate) are to be demonstrated the following variation of the technique is recommended :
(4) Stain in 1 per cent. Neutral Red, 30 seconds.
(5) Rinse quickly in water.
(6) Blot dry and allow the slide to dry completely in air (22° to 37°).
(7) Clear in xylene and mount in DPX.
With this variation the Neutral Red stains cytoplasmic as well as nuclear structures, concealing the tendency of Alcian Blue to stain connective tissues but leaving the acid mucopolysaccharide of the ground substance a deep blue-green.

THE EVAN'S BLUE METHOD FOR CONNECTIVE TISSUE GROUND SUBSTANCE (Perl and Catchpole, 1950)

Method

(1) Administer to selected animals, by the intravenous route, a suitable amount of 1·25 per cent. aqueous Evan's blue (4 ml. in the case of the guinea-pig).
(2) After 10 minutes kill the animals, preferably with chloroform or ether.
(3) Remove small blocks of tissue and immerse directly in *iso*pentane at —160°.
(4) Freeze-dry the tissues (see Appendix 3).
(5) Embed in soft paraffin (M.P. 40°).
(6) Cut thick sections (120 μ).
(7) Mount on albuminized slides.
(8) Remove wax with several changes of hot liquid paraffin.
(9) Mount in liquid paraffin and seal the edges of the coverslip with varnish.

Result

According to the authors, areas in which depolymerization has occurred, making the mucopolysaccharides of the ground substance more water soluble, are coloured blue by the dye.

OKAMOTO METHOD FOR GLUCOSE (after Muller, 1955-56)

Method

(1) Fix thin (2 mm.) tissue slices in methanol saturated with barium hydroxide for 24 hours at —10°.
(2) Dehydrate in three changes of absolute alcohol.
(3) Clear and embed in methyl benzoate, benzene, paraffin series.
(4) Cut 10 μ sections and mount on albuminized slides without contact with water.
(5) Place in paraffin oven at 56–58° until the wax melts.
(6) Remove wax with chloroform and bring to absolute alcohol.
(7) Immerse in alcoholic silver nitrate* for about 30 minutes.
(8) Wash repeatedly in 96 per cent. alcohol.
(9) Reduce in alcoholic formalin.†
(10) Bring through descending strengths of alcohol to water.
(11) Fix for 30 seconds in 5 per cent. sodium thiosulphate.
(12) Wash in distilled water, dehydrate, clear and mount in a suitable (non-reducing) resin.

Result

Black silver deposits indicate sites containing glucose.

METHODS FOR PECTIN

The Ruthenium Red Method

(*Paraffin sections ; various fixatives*)

(1) Bring sections to water.
(2) Treat with 25 per cent. alcoholic HCl for 24 hours at 22°.

* Add 90 ml. ethanol to 10 ml. of a 20 per cent. aqueous $AgNO_3$.
† Add 90 ml. ethanol to 10 ml. of 40 per cent. formaldehyde.

(3) Immerse in dilute (2 per cent.) ammonia for 4–6 hours.
(4) Transfer to 0·05 per cent. $RuCl_3$ and store in the dark until an adequate colour develops.

Result

A deep red colour indicates materials containing pectin. A similar colour is given by cellulose starch, inulin and lignin. The method is therefore quite unspecific.

The Krajčinović Amine Reaction

(*paraffin sections ; various fixatives*)

(1) Bring sections to water.
(2) Treat for 2 minutes in 1 per cent. HCl.
(3) Wash in three changes of absolute alcohol.
(4) Treat with 0·1 M-benzidine for 45 minutes.
(5) Wash in four changes of absolute alcohol.
(6) Diazotise by immersion for 1 minute in 0·1 N-HCl with 0·1 N-$NaNO_2$ (3 : 1).
(7) Wash in water.
(8) Couple with 0·1 M-β-naphthol.
(9) Wash in water and mount in glycerol.

Result

Pectin stains red. According to the author the method does not stain other polysaccharides.

FERRIC HYDROXAMIC ACID FOR ACETYLATED POLYSACCHARIDES (after McComb and McCready, 1957)

This method is best carried out on fresh frozen cold microtome sections which have been fixed briefly in alcohol or in Wolman's 5 per cent. acetic-ethanol.

Method

(1) Bring sections to absolute ethanol, remove and dry in air for a few minutes.
(2) Acetylate for 6 hours at 60° in 16 ml. acetic anhydride and 24 ml. dry pyridine.
(3) Wash in water.
(4) Treat for 5–10 minutes in hydroxylamine* (Appendix 12, p. 865).
(5) Wash in water.
(6) Treat with ferric perchlorate solution† for 5 minutes. (Colour should appear in $\frac{1}{2}$ to 1 minute).
(7) Wash in water, dehydrate, clear and mount in DPX.

* Dissolve 3·75 g. hydroxylamine hydrochloride in 100 ml. water and make alkaline with NaOH.

† Dissolve 1·93 g. of ferric chloride ($FeCl_3 . 6H_2O$) in 5 ml. conc. HCl. Add 5 ml. 70 per cent. perchloric acid and evaporate almost to dryness. Dilute to 100 ml. with water. This is the stock solution (stable 4 weeks at 0–4°). For use, add 8·3 ml. 70 per cent. perchloric acid to 60 ml. stock solution. Make up to 500 ml. with reagent grade methanol.

Result (Fig. 83)

Acetylated materials give an orange-red colour whose intensity is related to the number of reacting groups and therefore in some measure to the amount of polysaccharide glycol. The specificity of this method has not been tested.

CARMINE STAIN FOR GLYCOGEN (after Best, 1906)

(Bouin, Carnoy, alcohol, formation etc.; paraffin sections)

The best fixatives for the preservation of glycogens are either the ice-cold picro-alcohol formalin of Deane, Nesbett and Hastings (1946) or the mixture of Lison and Vokaer (1949) which is used at —73°. The second is composed of saturated picric acid in absolute alcohol (85 parts), formalin (10 parts) and acetic acid (5 parts). An acetone/CO_2 snow mixture is employed to cool the fixative and the smallest pieces of tissue possible are immersed therein.

Preparation of Solutions

Carmine Stock Solution. Add 2 g. carmine, 1 g. potassium carbonate and 5 g. potassium chloride to 60 ml. distilled water. Boil gently for 5 minutes, cool and filter. Add to the filtrate 20 ml. of ammonia (Sp. G. 0·880). This solution lasts 3 months at 0°– 4°.

Carmine Staining Solution. Dilute 15 ml. of stock solution with 12·5 ml. of ammonia (Sp. G. 0·880), and 12·5 ml. of methyl alcohol. This solution lasts for 2–3 weeks.

Best's Differentiator.

Absolute alcohol . .	8 ml.
Methyl alcohol . .	4 ml.
Aq. dest. . . .	10 ml.

Method

(1) Bring sections to absolute alcohol.
(2) Place sections in 1 per cent. celloidin in absolute alcohol/ether (equal parts) for 2 minutes.
(3) Dry in air.
(4) Pass through alcohol to water.
(5) Stain in Ehrlich's hæmalum for 5 minutes.
(6) Rinse and differentiate rapidly in 1 per cent. acid alcohol.
(7) Rinse in water.
(8) Stain in Best's carmine solution for 15–30 minutes.
(9) Differentiate in Best's differentiator, without previous rinsing, 5–60 seconds.
(10) Wash in 80 per cent. alcohol.
(11) Dehydrate in absolute alcohol, clear in xylene and mount in DPX.

Result (Figs. 26 and 27).

Nuclei, dark blue ; glycogen, red.

SILVER METHOD FOR ASCORBIC ACID (after Bourne, 1935, and Barnett and Bourne, 1941)

The original author advised the use of thin pieces of fresh tissue. If total ascorbic acid is required, small pieces of tissue are exposed to the vapour of acetic acid (5 minutes) and to H_2S (15 minutes), subsequently

removing the latter by exposure to a moderate vacuum for 10–30 minutes. This converts the oxidized to the reduced form, which alone reduces acid silver solutions.

Method

(1) Treat slices with acid silver nitrate solution (5 ml. glacial acetic acid to 100 ml. 5 per cent. aqueous silver nitrate, 5–10 minutes.
(2) Treat in the dark with 5 per cent. ammonia for 10–15 minutes.
(3) Wash in distilled water.
(4) Mount in glycerine.

Result

Black silver granules indicate the presence of reduced ascorbic acid.

SILVER METHOD FOR ASCORBIC ACID (after Bacchus, 1950)

Method

(1) Place tissues within a short while of removal in a dark vial containing 5 per cent. silver nitrate at pH 2–2·5. Incubate at 56° for 45–60 minutes.
(2) Pour off silver solution and add distilled water. Wash for 10–15 minutes.
(3) Pour off distilled water and add 5 per cent. sodium thiosulphate. Leave for 30–45 minutes.
(4) Dehydrate in dioxan.
(5) Embed in paraffin wax.
(6) Cut sections 6–10 μ thick and mount on slides.
(7) Bring to water, counterstain with hæmalum.
(8) Wash, dehydrate in alcohol, clear in xylene, mount in Canada balsam.

Result

Black granules indicate ascorbic acid.

SILVER METHOD FOR ASCORBIC ACID (after Jensen and Kavaljian, 1956)

(freeze-dried ; paraffin sections)

Method

(1) 10 μ sections, dry mounted on to albuminized slides, are incubated at 37–40° overnight.
(2) Without removing paraffin place sections in 10 per cent. $AgNo_3$ in 3 per cent. acetic acid for 8–14 hours.*
(3) Wash in 95 per cent. alcohol and dehydrate in absolute alcohol.
(4) Remove paraffin wax with xylene.
(5) Mount in Permount or suitable non-reducing resin.
The finished preparations should be stored in the dark.

Result

Reduced silver granules indicate ascorbic acid.

* A modified method for reduction of dehydroascorbic acid was used before this stage, when appropriate, as follows :
Place sections in an atmosphere of H_2S for 15 minutes. Pass O_2-free nitrogen over the sections for a further 15 minutes.

SILVER METHOD FOR GLYCOGEN AND MUCIN* (Gomori, 1946, modified by Arzac and Flores, 1949)

(Bouin or alcohol ; paraffin sections)

Preparation of Reagents

Lithium-silver Solution.

10 per cent. aqueous $AgNO_3$ . .	4 ml.
Sat. aqueous Li_2CO_3 . . .	16 ml.

Add conc. ammonium hydroxide (Sp. G. 0·880) drop by drop until the lithium silver complex is almost dissolved, leaving a light turbidity.

Add sat. aqueous Li_2CO_3 to make up 100 ml.

Filter and keep in well-stoppered dark bottles at 4°.

Use at full strength (if formalin reduction is employed) or diluted 1 : 10 (if no formalin reduction).

Piperazine-silver Solution.

0·5 per cent. piperazine . . .	100 ml.
10 per cent. aqueous $AgNO_3$ . .	1 ml.

For use, dilute 1 : 4 with distilled water and add 2 drops of sat. aqueous Li_2CO_3 to each 50 ml.

Method

(1) Bring sections to alcohol and cover with 1 per cent. celloidin, dry and pass through alcohol to water.

(2) Immerse in 10 per cent. chromic acid, 20–30 minutes.

(3) Wash in running water and then in distilled water.

(4) Treat with dilute lithium-silver or piperazine-silver at 45° for 15–60 minutes. Alternatively, use stock lithium-silver at 17°–22° for the same period.

(5) After the first two silver solutions wash in water ; after the third in 2 per cent. neutral formalin for 30–60 seconds.

(6) Tone with 1 : 500 gold chloride if necessary.

(7) Rinse in 5 per cent. sodium thiosulphate, 3–5 minutes.

(8) Wash in water and remove celloidin with acetone.

(9) Dehydrate, clear and mount as usual.

Result

Glycogen and mucin, brown to black. Background colourless.

* This technique is not specific for glycogen but it is included for its general usefulness as an aldehyde method (see Chapter XI).

APPENDIX 10

METACHROMATIC METHODS FOR AMYLOID

(I) Dahlia Method (Lendrum, 1951)

(*Formalin or alcohol; paraffin sections, fresh frozen, alcohol-fixed sections*)

Method

(1) Bring paraffin sections to water.

(2) Stain in 1 per cent. aqueous methyl violet or 1 per cent. aqueous dahlia, 3 minutes.

(3) Differentiate in 70 per cent. formalin (commercial formalin, 7 parts; water, 3 parts).

(4) Rinse well and flood with saturated aqueous sodium chloride, 5 minutes.

(5) Rinse well and mount in corn syrup.

Result

Amyloid, pink or red. Other structures blue or violet. According to the author this method gives preparations which remain stable for a number of years.

(II) Modified Methyl Violet Method

(*Various types of preparation can be employed; fresh frozen sections briefly fixed in 5 per cent. acetic-ethanol are recommended*)

Method

(1) Stain mounted sections for 12 or more hours at 22° in 40 ml. distilled water containing one drop of freshly filtered 1 per cent aqueous methyl violet.

(2) Differentiate quickly in dilute acetic acid (4 drops per 100 ml.).

(3) Immerse in saturated aqueous sodium chloride, 5 minutes.

(4) Mount in glycerine jelly.

Result

Amyloid deposits stain pink or red.

MODIFIED CONGO RED METHOD

(*All types of material; fresh frozen sections briefly fixed in acetic-ethanol are recommended*)

Method

(1) Stain in 1 per cent. aqueous Congo red for 1–6 hours.

(2) Treat with 1 per cent. KI for 60 seconds.

(3) Differentiate in 70 per cent. alcohol until only amyloid deposits are stained (use control slide if necessary).

(4) Wash in water.

(5) Counterstain nuclei with Mayer's hæmalum 1½ to 3 minutes.

(6) Differentiate for a few seconds in 1 per cent. acid alcohol.

(7) Blue in dilute buffer (pH 7–8).

(8) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic resin.

Result

Amyloid stains brick-red, nuclei blue.

REDUCING METHOD FOR AMYLOID (King, 1948)

(Formalin; frozen sections)

Preparation of Silver Solution

Add ammonia drop by drop to 5 ml. of 10 per cent. $AgNO_3$ to produce and dissolve the precipate. Add 6·8 ml. 3·5 per cent. sodium carbonate (anhydrous) and 75 ml. distilled water. For use take 10 ml. of this solution and add a few drops of pyridine.

Method

(1) Cut thin frozen sections (10–15 μ or less).
(2) Warm 10 ml. of silver solution to about 40° and immerse sections after brief rinsing to remove excess formalin. Leave until sections are brown.
(3) Wash in 5 per cent. sodium thiosulphate, 3 minutes.
(4) Rinse in water.
(5) Dehydrate in 50 per cent. alcohol for 5–10 minutes.
(6) Pass rapidly through 70–95 per cent. alcohol.
(7) Clear in carbol xylene (Phenol, 1 part; xylene, 3 parts).
(8) Lift sections on to clean slides and mount in Canada balsam.

Result

Amyloid blackish-brown. Some other substances may be stained brown, particularly those containing reducing lipids.

APPENDIX 11

EXTRACTIVE METHODS FOR LIPIDS

Baker's Pyridine Extraction Test

Method

(1) Fix in a weak solution of Bouin's fixative (sat. aqueous picric acid 50 ml., commercial formalin 10 ml., glacial acetic acid 5 ml., distilled water 35 ml.) 20 hours.

(2) Wash in alcohol to remove picric acid.

(3) Immerse in pyridine at 17°–22°, 30 minutes.

(4) Immerse in pyridine at 60°, 24 hours.

(5) Wash in running water, 2 hours.

(6) Transfer to the dichromate-calcium mordant of the acid hæmatein method (q.v.).

Result

All lipids should be removed by the pyridine extraction procedure which can be followed by Sudan black B or other colorant methods as well as by the acid hæmatein method.

Keilig's Extractions

Thin, unfixed, blocks of tissues are treated with the four solvents, cold acetone, hot acetone, hot ether and hot chloroform/methanol (equal parts). Three changes of each of the solvents should be used, for 3, 3 and 12 hours respectively. For the hot solvents a Soxhlet extractor may be used, or a simpler apparatus if desired. After extraction, the blocks are washed in water and frozen sections are stained in Sudan black B in 70 per cent. alcohol. Dispersion effects are marked so that localization is inaccurate. Cold acetone, for instance, disperses certain phospholipids from the cells containing them and hot acetone invariably does so. They remain in the section, however, and stain with Sudan black.

BROMINATION TECHNIQUES (Lillie, 1954)

Paraffin Sections

(1) Remove wax with xylene.

(2) Wash in two changes of carbon tetrachloride.

(3) Brominate for one hour in 39 ml. CCl_4 containing 1 ml. of bromine. (Control sections should be exposed to the solvent alone.)

(4) Wash in two changes of carbon tetrachloride.

(5) Transfer to 95 per cent. alcohol, 70 per cent. alcohol and bring to water.

(6) Carry out selected technique.

Result

Ethylene groups (C = C, double bonds) no longer react with oxidizing agents to form aldehydes.

Frozen Sections

(1) Attach sections to slides, blot lightly and dry for 10–15 minutes.
(2) Immerse in freshly made bromine water (1 ml. bromine, 39 ml. water) for 1–6 hours.
(3) Wash in water.
(4) Treat with 0·5 per cent. sodium metabisulphite for 1–2 minutes, to remove free bromine.
(5) Wash in running water.
(6) Carry out selected technique.
This method is applicable to studies of solvent-labile fats.

SECONDARY FLUORESCENCE METHODS

Aqueous Phosphine 3 R (Popper, 1944)

(*Formalin or formol-calcium; frozen sections*)

Method

(1) Formalin or formol-ealcium fixation is preferable. Frozen seetions 10 μ thick are cut.
(2) Stain sections for 3 minutes in 0·1 per cent. aqueous Phosphine 3 R.
(3) Rinse briefly in water.
(4) Mount in 90 per cent. glycerine.
(5) Examine by ultraviolet light.

Result

Fatty acids, soaps and cholesterol negative. Other lipids, ineluding cholesterol esters, give a silvery-white fluorescence.

3 : 4-Benzpyrene (Berg, 1951)

(*Formalin or formol-calcium; frozen sections*)

Preparation of the Staining Solution

Prepare a saturated solution of caffeine in water at room temperature (about 1·5 per cent.). To 100 ml. of filtered solution add 0·002 g. 3 : 4-benzpyrene and incubate at 37° for 2 days. Filter off excess benzpyrene and dilute with an equal volume of distilled water. Allow to stand for 2 hours and refilter. The solution eontains about 0·00075 g./100 ml. and it can be used for some months if stored in the dark in a well-stoppered bottle.

Method

(1) Cut frozen sections at 10 μ and stain for 20 minutes in the benzpyrene solution.
(2) Wash in water and mount the sections on slides.
(3) Examine in water by ultraviolet light.

Result (Fig. 45)

Lipids, including the finest lipid granules, give a blue or bluish-white fluorescence. Since this fades relatively rapidly, permanent sections cannot be obtained.

METHODS FOR SUGAR-CONTAINING LIPIDS

Modified Molisch Reaction (after Diezel, 1954)

(*Formalin; frozen or paraffin sections*)

Method

(1) Apply to the dry section, mounted on a slide and after removal of wax if necessary, 2–3 drops of a mixture containing 0·5 ml. 5 per cent. alcoholic α-naphthol and 10 ml. of 2N-H_2SO_4.*

(2) Place section in an incubator at 90°–100° (without coverslip), or on a hot stage at the same temperature.

Result

Cerebrosides and gangliosides stain violet-red. The colour is not stable and fades to grey after 1 hour.

Modified Brückner Reaction (after Diezel, 1954)

(*Formalin; frozen or paraffin sections*)

Method

(1) Apply to mounted, dry sections, after removal of wax if necessary, 2–3 drops of a mixture of 1 ml. 2 per cent. aqueous orcinol (3,5-dihydroxytoluene) and 10 ml. 2N-H_2SO_4.

(2) Place section in an incubator or on a hot stage at 90°–100°.

Result

Cerebrosides and gangliosides stain red to bluish red.

Modified Roe-Rice Method for Pentoses

(*Formalin; frozen or paraffin sections*)

Method

(1) Immerse mounted sections for 5–10 minutes at 70°–85° in acetic-bromoaniline.†

Result

Pentoses stain red within a few minutes.

THE ACID HÆMATEIN METHOD (Baker, 1946)

(*Formol-calcium, post-chromation; frozen sections*)

Preparation of Solutions

(1) **Formol Calcium Fixative.**

40 per cent. formalin . . .	10 ml.
10 per cent. $CaCl_2$ (anhydrous) aqueous solution. . . .	10 ml.
Distilled water	80 ml.

To this is added a piece of chalk to maintain neutral pH.

* Diezel recommends the addition of one drop of some convenient wetting agent (Wasa ,Teepol) to the section to ensure proper wetting of the tissues.

† Saturated 100 ml. of acetic acid with 4 g. of thiourea. Allow sediment to settle and pour off supernatant. Add to this 2 g. bromoaniline. Stored in a dark bottle this solution is stable for about 7 days.

(2) **Dichromate-calcium.**

Potassium dichromate . . .	5 g.
$CaCl_2$ (anhydrous)	1 g.
Distilled water	100 ml.

(3) **Acid Hæmatein.** Place 0·05 g. hæmatoxylin in a flask, add 48 ml. distilled water and exactly 1 ml of 1 per cent. $NaIO_4$. Heat until the water begins to boil, cool and add 1 ml. of glacial acetic acid. (The author recommended B.D.H. reagent hæmatoxylin.) This solution should be used on the day of preparation.

(4) **Borax-ferricyanide Differentiator**

Potassium ferricyanide . . .	0·25 g.
Sodium tetraborate, 10 H_2O . .	0·25 g.
Distilled water	100 ml.

This solution should be kept in the dark.

Method

(1) Fix small blocks of tissue in formol-calcium, 6–18 hours.
(2) Transfer to dichromate-calcium for 18 hours at 22°.
(3) Transfer to dichromate-calcium for 24 hours at 60°.
(4) Wash well in distilled water.
(5) Cut frozen sections at 10 μ (embed in gelatine if necessary).
(6) Mordant in dichromate-calcium for 1 hour at 60°.
(7) Wash in distilled water.
(8) Transfer to acid hæmatein solution for 5 hours at 37°.
(9) Rinse in distilled water.
(10) Transfer to borax-ferricyanide for 18 hours at 37°.
(11) Wash in water.
(12) Mount in glycerine jelly.

Result (Fig. 88)

Nucleoprotein and phospholipins dark blue or blue-black. Mucin dark blue, fibrinogen pale blue. Cytoplasm pale yellow.

N.B. A positive result for phospholipins with this technique should be accompanied by a negative reaction after pyridine extraction. In the absence of such a negative extraction test the dark blue or blue-black staining material is not phospholipin.

CONTROLLED CHROMATION (Elftman, 1954)

This method is normally applied to thin blocks of tissue which, after chromation is completed, are dehydrated and embedded in paraffin. These blocks are brittle and sections must usually be cut at 8 μ. Elftman also applied the method to frozen sections, remarking that the inevitable increase in fragility presented a problem. The use of fresh, cold microtome, sections mounted on slides or coverslips entirely overcomes this problem and the sections can be as thin as 2 μ if required.

Method

(1) Treat blocks of fresh tissue (or fresh frozen sections) with 2·5 per cent. potassium dichromate adjusted to pH 3·5 with 0·2 M-acetate buffer. The average time of chromation is 18 hours at 56°–60°.

(2) Wash in water. Sections are ready for subsequent treatment at this stage.

(3) Dehydrate blocks in ascending strengths of alcohol, clear in benzene and embed in paraffin. Cut sections 8–10 μ thick.

(4) Stain pairs of sections, one in Sudan black B in propylene glycol (p. 855) and the other in 0·1 per cent. hæmatoxylin in 0·2 M-acetate buffer at pH 3·0, containing 0·005 per cent. potassium ferricyanide (2 hours, 56°).

Result

Chromated lipids are stained by both Sudan black and hæmatoxylin; non-chromated lipids only by Sudan black; non-lipids only by hæmatoxylin.

Many other methods given in Appendix 11 can be applied to sections after controlled chromation. Especially useful is the copper phthalocyanin technique (p. 857).

SUDAN BLACK B STAINING FOR LIPIDS IN PARAFFIN SECTIONS (after McManus, 1946)

(*Various fixatives; paraffin sections*)

McManus advised fixation of tissues for 1–5 weeks in a solution of 1 g. cobalt nitrate in 80 ml. distilled water with 10 ml. 10 per cent. $CaCl_2$* and 10 ml. commercial (40 per cent.) formalin. Subsequently, as an optional step, he recommended post-chroming for 24–48 hours in 3 per cent. $K_2Cr_2O_7$.

The blocks were dehydrated in 3 changes of acetone, each of $\frac{1}{2}$ hour duration, and placed directly in molten paraffin wax. This procedure seems to have no advantage over the routine methods of embedding, especially if post-chroming has been carried out.

Method

(1) Bring sections to 70 per cent. alcohol.

(2) Stain for 30 minutes at room temperature in saturated Sudan black B in 70 per cent. alcohol (longer times are advisable in the case of tissues fixed other than in formol-calcium and for tissues which have not been post-chromed. I employ up to 16 hours at 60° for resistant cases).

(3) Remove excess dye by rinsing quickly in 70 per cent. alcohol.

(4) Wash in running water.

(5) Counterstain in Mayer's carmalum 16 hours, or in 1 per cent. aqueous neutral red, 1 minute.

(6) Wash in water and mount in glycerine jelly.

Result

Lipids stain black or blue if present in sufficient quantity. Even a brownish-black stain may be an indication of the presence of lipid or lipoprotein. Nuclei, red.

SUDAN BLACK B METHOD FOR MASKED LIPIDS (Ackerman, 1952)

(*Blood smears*)

Method

(1) Fix in formalin vapour for 2–5 minutes.

(2) Immerse films in 25 per cent. acetic acid (or 5 per cent. citric, 10 per cent. oxalic or 20 per cent. formic acids) for 2 minutes.

* Lillie advised calcium acetate in place of the chloride, to make a final strength of 2 per cent.

(3) Wash thoroughly in tap water, then in distilled water and allow films to dry.

(4) Stain in saturated Sudan black B in 70 per cent. ethanol (this solution must be at least one week old).

(5) Differentiate in 70 per cent. alcohol.

(6) Blot dry and mount in glycerine jelly.

Result

Bound lipids and lipids surviving the staining procedure stain black.

ACETONE-SUDAN BLACK METHOD FOR BOUND LIPIDS (after Berenbaum)

(*Formalin, Zenker, Carnoy, etc.; paraffin sections*)

Method

(1) Wash sections overnight in running water after first bringing to water in the usual manner.

(2) Rinse in absolute acetone.

(3) Stain in 2 per cent. Sudan black B in acetone at 37° for 1–24 hours.

(4) Wash in xylene till no further stain comes away (5–10 minutes).

(5) Mount in Canada balsam or in a suitable synthetic medium.

Result

Chromatin, nucleoli and various bound lipids stain black. Neutrophil granules, platelets, amyloid and fibrin also stain.

BURNT SUDAN BLACK METHOD (after Berenbaum)

This method is carried out as above except that, instead of stage 3, sections are placed on a rack and covered with saturated Sudan black B in 70 per cent. alcohol. This is set alight and allowed to burn off. The process is repeated 6–12 times. Sections are then washed in 70 per cent. alcohol, cleared in xylene and mounted.

Result

As with the acetone-Sudan black method except that red cells, eosinophil granules and myelin sheaths also stain.

THE PHOSPHOMOLYBDIC ACID METHOD (Landing *et al.*, 1952)

(*Formalin or formol-calcium ; frozen sections*)

Method

(1) Place section on a gelatinized slide, drain, blot, and expose to formalin vapour for 10–15 minutes.

(2) Allow the section to dry thoroughly and dip into 50/50 acetone-ether.

(3) Transfer slide to 1 per cent. phosphomolybdic acid in 50/50 ethanol-chloroform for 15 minutes.

(4) Rinse in ethanol-chloroform, then in chloroform and dry.

(5) Dip slide into 1 per cent. aqueous stannous chloride in 3N-HCl (freshly made).

(6) Wash in water.

(7) Counterstain with 1 per cent. aqueous eosin.
(8) Dehydrate, clear and mount in Canada balsam.

Result

Choline-containing lipids stain in molybdenum blue shades. This method can be applied to fresh, cold microtome, sections.

OKAMOTO'S MERCURY DIPHENYLCARBAZONE METHOD (after Ueda)

(*Formalin; frozen sections*)

Method (so-called Common Method)

(1) Cut 6–10 μ sections and place in acetone containing 0·65 per cent. magnesium chloride for 48 hours at 22°.
(2) Rinse thoroughly in water.
(3) Place sections in mercuric nitrate solution at 0°–4° for 24 hours. (Saturated mercuric nitrate in 60 per cent. alcohol, 10 ml. ; 0·2 per cent. NaCl, 0·2 ml.)
(4) Wash thoroughly in water.
(5) Treat with 5 per cent. KI for 4–5 minutes. (The colour of the sections changes to reddish-yellow ; after 2–3 minutes this colour is discharged.)
(6) Wash in water for 10 minutes.
(7) Treat with 2 per cent. sodium acetate for 10 minutes.
(8) Place in saturated diphenylcarbazone in 90 per cent. alcohol, 10 minutes.
(9) Wash thoroughly in water and mount in glycerine jelly.

Result (Fig. 89)

Phospholipids appear bluish-violet. Cerebrosides also stain.

The original authors stated that not only phospholipids and cerebrosides were positive but also "some other materials besides lipids." They endeavoured to distinguish between phospholipids and cerebrosides by means of cold pyridine extraction for 48 hours, carried out *after* the staining procedure.

The so-called separation test methods were designed to demonstrate sphingomyelin.

Method A

(1) Rinse sections (formalin-fixed, frozen) in acetone.
(2) Place in ether for 48 hours at room temperature.
(3) Rinse thoroughly in acetone.
(4) Apply stages 2–9 of the common method.

Result

Sphingomyelin only remains positive.

Method B

(1) Apply stages 1–3 of the common method.
(2) Rinse in acetone.
(3) Treat with ether for 48 hours.
(4) Rinse in acetone and wash in water.
(5) Apply stages 5–9 of the common method.

Result

Sphingomyelin and lecithin are positive.

These extractions have been shown by Edgar and Donker (1957) to produce much less clear cut results when tested by chemical procedures than the original authors suggest. They should only be used, with extreme caution in interpretation, as pointers to the development of more satisfactory methods.

MARCHI METHOD FOR DEGENERATING MYELIN (after Swank and Davenport)

(*Formalin*)

Method

(1) Fix thin (3 mm.) tissue slices in 10 per cent. formalin for 48 hours.

(2) Transfer in solution containing 1 per cent. aqueous potassium chlorate, 60 ml., 1 per cent. aqueous osmium tetroxide, 20 ml., acetic acid, 1 ml., formalin, 12 ml. (About 15 volumes of this mixture to 1 volume of tissue is adequate.)

(3) Shake every day and turn the tissues over. Keep in the OsO_4 solution for 7–10 days, in the dark, in a vapour-tight container.

(4) Wash in running water for 48 hours.

(5) Dehydrate in 70 per cent. and 95 per cent. alcohol, transfer via absolute alcohol and alcohol/ether (1 : 1) to 1 per cent. celloidin in alcohol/ether.

(6) Cut sections 30–60 μ thick and mount in Canada balsam.

Glees (1943) recommends in place of stage 5 that 60–90 μ sections should be cut on the freezing microtome and Smith (1956) states that all kind of artifacts are much less in frozen sections.

Result

Degenerating myelin products black ; background yellow or brown.

MODIFIED MARCHI METHOD FOR PARAFFIN SECTIONS (Wolman, 1957)

(*Alcohol or formalin; paraffin sections*)

Method

(1) Fix small pieces of tissue in 95 per cent. alcohol for 4 days.

(2) Dehydrate in absolute alcohol, clear in xylene and embed in paraffin.

(3) Cut 8 μ sections and transfer to 1 per cent. OsO_4 in carbon tetrachloride for 24 hours.

(4) Wash in three changes of carbon tetrachloride.

(5) Transfer to xylene (two changes).

(6) Mount in a suitable synthetic resin.

Result

Degenerating myelin brown to black.

METHODS USING COLORANT DYES IN SIMPLE SOLUTION

Sudan III and IV Method for Neutral Fats (after Kay and Whitehead, 1941)

(*Formalin; frozen sections*)

Preparation of the Staining Solution

Mix equal quantities of dry Sudan III and Sudan IV and place in a clean dry bottle. Fill bottle with Herxheimer's mixture (equal parts of

acetone and 70 per cent. alcohol) and shake well. Leave solution for a few days until saturated. For use, pipette off some of the supernatant. Use in a closed vessel to avoid evaporation and precipitation of the stain.

Method

(1) Cut frozen sections at 10 μ into distilled water, or into 1 per cent. formalin if storage is necessary.

(2) Rinse in 70 per cent. alcohol.

(3) Stain for not more than 1 minute in the Sudan mixture, taking care to avoid letting the section fold over.

(4) Rinse in 50 per cent. alcohol to remove excess stain.

(5) Rinse in water, holding section under until all alcohol has diffused out.

(6) Counterstain in dilute (1 : 4) Ehrlich's hæmatoxylin for 1–3 minutes.

(7) Differentiate, if necessary, in 0·5 per cent. HCl in 50 per cent. alcohol.

(8) Wash in distilled water to which a few drops of ammonia have been added.

(9) Mount in glycerine jelly.

Result

Lipids (neutral fats), orange-red to orange. Nuclei, blue.

The main disadvantages of this method are : (1) there is a tendency to extraction of fine lipid droplets ; (2) the colour imparted to the remaining fine lipid droplets is not sufficiently strong ; and (3) the dyes have a marked tendency to form crystalline precipitates on storage.

The Oil Red O Method for Neutral Fats (after Lillie, 1944)

(*Formalin; frozen sections*)

Preparation of the Stain

Prepare the stock solution by adding about 0·5 g. Oil Red O to 100 ml. 98 per cent. *iso*propanol. For use, dilute 6 ml. with 4 ml. of water, stand for 24 hours and filter. Use this filtrate as a stock staining solution, filtering through Whatman No. 42 paper, as necessary, a sufficient amount for immediate use.

Lillie (1945a and b) also recommended the dyes Oil Blue N and Coccinel Red for fat staining from *iso*propanol solutions.

Method

(1) Stain frozen sections, after rinsing in water and then in 60 per cent. *iso*propanol, in freshly filtered Oil Red solution for 10 minutes.

(2) Differentiate briefly in 60 per cent. *iso*propanol (keep tightly stoppered or make up freshly).

(3) Wash in water.

(4) Stain for 5 minutes in Mayer's hæmalum.

(5) Wash in running water for at least 10 minutes.

(6) Mount in glycerine jelly or gum syrup.

Result

Lipids, red. Nuclei, blue.

This method has two advantages over the Sudan III and IV method. First, the colour is deeper and the staining of smaller droplets can therefore be appreciated ; secondly, there is less tendency to formation of dye precipitates.

The Fettrot Method for Neutral Fat

(Formalin; frozen sections)

Method

(1) Cut frozen sections 10–15 μ, mount on egg-albuminized slides and dry thoroughly in air to ensure adherence.
(2) Stain in Meyer's hæmalum 4–6 minutes.
(3) Wash in running water for 30 minutes.
(4) Rinse quickly in 50 per cent. alcohol.
(5) Stain in a saturated solution of Fettrot 7B (Ciba) 10–15 minutes.
(6) Wash in water.
(7) Mount in glycerine jelly.

Result

Lipids, pinkish-red. Nuclei, dark blue.

Fettrot 7B or Technical Fettrot are available from Messrs. Ciba Ltd. This method gives very beautiful and permanent results, since unlike the red Sudan dyes and Oil Red O, Fettrot has no tendency to crystallize when used from alcoholic solutions, and mounted specimens retain their clarity over a long period.

The Propylene Glycol-Sudan Method for Lipids (after Chiffelle and Putt, 1951)

(Formalin, dichromate, etc.; frozen or paraffin sections)

Preparations of Dye Solutions

Dissolve 0·7 g. Sudan IV or Sudan black B in 100 ml. pure propylene glycol by heating to 100° and stirring for a few minutes. Filter through Whatman No. 2 filter paper. Cool to room temperature and filter through glass wool by means of a suction filter. (I have used Fettrot in place of the Sudan dyes, with excellent results.)

Method

(1) Cut frozen sections 10–15 μ, wash well to remove formalin.
(2) Mount sections on albuminized slides and dry at room temperature.
(3) Transfer to the staining solution for 5–7 minutes, with occasional agitation throughout this period.
(4) Transfer to 85 per cent. propylene glycol for 2–3 minutes, with agitation.
(5) Transfer to 50 per cent. propylene glycol for 2–3 minutes.
(6) Wash gently in warm running water for 1 minute.
(7) Counterstain in Mayer's hæmalum (if using Sudan IV or Fettrot), 4–6 minutes.
(8) Wash well in water and mount in glycerine jelly.

Result

Lipids are stained red or black, depending on the dye used. The method has the advantage of considerable latitude in the time of differentiation after staining. Only if differentiation is carried out for 20 minutes or so will the dyes begin to be removed from solution in tissue lipids. Phosphatides as well as neutral fats are often strongly stained. With the Sudan IV and Fettrot solutions a tendency to the formation of crystalline deposits is noted.

The Nile Blue * Method for Neutral and Acidic Lipids (after Cain, 1947)

(*Formol-calcium; frozen sections*)

Method

(1) Fix small blocks of tissue in Baker's formol-calcium fixative.
(2) Treat with dichromate-calcium (Baker) in the two stages of the acid hæmatein method. This step is optional.
(3) Cut frozen sections at 10 μ.
(4) Stain one section (A) in saturated Sudan black B in 70 per cent. alcohol as a control for lipids.
(5) Stain one section (B) in 1 per cent. aqueous Nile Blue at 60°, 5 minutes.
(6) Wash quickly in water at 60°.
(7) Differentiate in 1 per cent. acetic acid at 60°, 30 seconds.
(8) Stain another section (C) as section (B) and restain in 0·02 per cent. Nile Blue at 60°.
(9) Wash and differentiate section (C) as section (B) (stages 6 and 7).
(10) Mount all sections in glycerine jelly.

Comment

Only purely lipid inclusions can be considered. An inclusion can be established with certainty as purely lipid only by staining a pyridine-extracted control section with Sudan black B and comparing the result with formol-calcium-fixed, unextracted, sections stained in the same way. If there is no difference between sections (B) and (C) the first may be discarded, since what will stain with 1 per cent. Nile Blue will stain with 0·02 per cent.

Result

Neutral lipids, red or pink. Acidic lipids, blue.

NILE BLUE METHOD FOR PHOSPHOLIPIDS (Menschik, 1953)

(*Formol-calcium; frozen sections*)

Method

(1) Fix for 6–12 hours and cut frozen sections without embedding in gelatin or carbowax.
(2) Stain for 90 minutes at 60° in saturated aqueous Nile blue sulphate 500 ml. with 50 ml. 0·5 per cent. H_2SO_4 (boil for 2 hours before use).
(3) Rinse in distilled water.
(4) Place in acetone heated to 50°.
(5) Remove acetone from source of heat and allow sections to remain in it for 30 minutes.
(6) Differentiate in 5 per cent. acetic acid 30 minutes.
(7) Rinse in distilled water.
(8) Differentiate again in 0·5 per cent. HCl for 3 minutes.
(9) Wash in distilled water and mount in glycerine jelly.

Result

Phospholipids blue.

* The term Nile Blue is used to mean the commercial dye which is a mixture of the oxazine sulphate (true Nile Blue) and the oxazone (Nile Red).

THE COPPER PHTHALOCYANIN METHOD (after Klüver and Barrera, 1953)

(*Formalin or formol-calcium; frozen sections; various; paraffin sections*)

Method

(1) Bring sections to absolute alcohol.
(2) Stain in 0·1 per cent. Luxol Fast blue MBS (Dupont) or Methasol Fast blue 2 G (I.C.I. Ltd.) in 95 per cent. alcohol for 6–18 hours at 56°–60°.
(3) Rinse in 70 per cent. alcohol and wash in water.
(4) Differentiate in 0·05 per cent. aqueous lithium carbonate for ½–2 hours (90 minutes for formalin-fixed frozen sections).
(5) Rinse in water.
(6) Counterstain if required either in Mayer's carmalum 10–30 minutes or in 1 per cent. aqueous Neutral red (1–30 minutes).
(7) Rinse in water and dehydrate in 70 and 95 per cent. alcohol.
(5) Clear in xylene and mount in a suitable synthetic resin.

Result (Figs. 90 and 91)

Phospholipids except sphingomyelin stain blue. So do gangliosides and probably cerebrosides.

Sphingomyelin can be stained by substituting chloroform for ethanol as the dye solvent (stage 2) but many other tissue components also stain if this is done.

The method is suitable for application to fresh, cold microtome, sections, treated by Elftman's controlled chromation (p. 849).

METHOD USING COLORANT DYES IN COLLOIDAL SUSPENSION

The Gelatine-Sudan Method (after Govan, 1944)

(*Formalin; frozen sections*)

Preparation of the Suspension

Make a saturated solution of Sudan III and Sudan IV in acetone and add, drop by drop, to a 1 per cent. aqueous gelatine solution containing 1 per cent. acetic acid until a brick-red milky fluid is obtained after constant stirring. Place the mixture in the 37° incubator to evaporate the acetone (2 hours). Filter through coarse paper.

Method

(1) Cut frozen sections at 10 μ and transfer to 1 per cent. gelatine 2–3 minutes.
(2) Stain for 30 minutes in the suspension.
(3) Wash in 1 per cent. gelatine for 2–3 minutes.
(4) Stain in Mayer's hæmalum 4–6 minutes.
(5) Blue in water with added ammonia.
(6) Mount in glycerine jelly or in Karo corn syrup.

Result (Fig. 87)

Lipids, including the smallest droplets, orange-red. Nuclei, blue.

FISCHLER'S METHOD FOR FATTY ACIDS

(*Formalin; frozen sections*)

Preparation of Solutions

(1) **Weigert's Lithium-hæmatoxylin.** *Solution A.* Ten per cent. hæmatoxylin in absolute alcohol.

Solution B. Sat. aqueous lithium carbonate 10 ml., distilled water 90 ml.

For use mix one part each of solutions A and B.

(2) **Weigert's Borax-ferricyanide.**

Borax ($Na_2B_4O_7$, $10H_2O$) . .	20 g.
Potassium ferricyanide . . .	25 g.
Distilled water	1,000 ml.

Method

(1) Mordant 10 μ frozen sections for 24 hours at 37° in saturated aqueous cupric acetate.

(2) Wash in distilled water.

(3) Stain for 20 minutes in Weigert's lithium-hæmatoxylin.

(4) Differentiate in borax-ferricyanide until the red cells are no longer dark blue.

(5) Wash in distilled water and mount in glycerine jelly.

Control sections should be stained which have previously been extracted for 24 hours with 3 changes of alcohol/ether (equal parts).

Result

Tissue components staining dark blue in the unextracted and colourless in the extracted sections are presumed to contain fatty acids. For interferences see text.

OKAMOTO'S METHOD FOR FATTY ACIDS (after Ueda)

(10 *per cent. formol-saline; frozen sections*)

Method

(1) The fixative should contain at least 20 per cent. NaCl.

(2) Cut frozen sections and wash in 20 per cent. NaCl.

(3) Transfer sections to copper solution for 24 hours (saturated aqueous $Cu(NO_3)_2.H_2O$, 0·5 ml., distilled water 100 ml.).

(4) Alternatively, transfer to an alcoholic copper solution (sat. aq. $Cu(NO_3)_2.H_2O$, 0·2 ml., 75 per cent. ethanol, 100 ml.) for 2–3 hours.

(5) Wash thoroughly in water.

(6) Treat with alcoholic *p*-dimethylaminobenzylidene rhodanine * for 24 hours at 58°.

(7) Washing in water.

(8) Mount in glycerine jelly.

Result

Fatty acids and their alkaline earth salts stain reddish-brown.

* Saturated alcoholic *p*-dimethlaminobenzylidene rhodanine 5 ml., Absolute ethanol containing 2 per cent sodium acetate, 95 ml.

FEYRTER'S "MOUNTING-STAINING" METHOD FOR ACIDIC LIPIDS

(*Formalin; frozen or cold microtome sections*)

Preparation of Staining Solutions

Dissolve 1 g. of either thionin or cresyl violet in 0·5 per cent. aqueous tartaric acid. Not all samples of these dyes give equally good staining.

Method

(1) Cut frozen sections (10–15 μ) and wash these thoroughly in water until free from all but closely bound formalin.
(2) Mount on clean slides from distilled water and blot dry.
(3) Cover with staining solution and apply a coverslip.
(4) Remove excess stain with filter paper until the edges of the coverslip are dry.
(5) Seal the coverslip with molten paraffin wax.
(6) Allow to stand at room temperature and examine from time to time.
(If rapid results are required, ring the coverslip with vaseline and incubate the slide at 60° for 15–30 minutes.)

These preparations, sealed with paraffin wax, are stable for years.

Result

A fine red or pink metachromasia is given by epithelial mucins containing acid mucopolysaccharide and by connective tissue mucin and mast cell granules. Myelin sheaths are bright rose-red in colour and phosphatides and cerebrosides are a similar, usually less intense, rose-red. This method must always be controlled by the use of other tests. Baker's acid hæmatein and the Sudan black methods for instance.

THE U-V SCHIFF METHOD (after Belt and Hayes)

(*Cold formalin; frozen sections. Cold microtome*)

Method

(1) Fix sections for 12–18 hours in cold 10 per cent. neutral formalin.
(2) Cut sections and place under a source of ultraviolet light for 2–4 hours.
(3) Treat with Schiff's reagent for 15 minutes.
(4) Rinse in three changes of sulphurous acid water.
(5) Rinse in distilled water.
(6) Mount in glycerine jelly.

Result

A magenta-red colour absent from unirradiated control sections indicates unsaturated lipids.

This method produces less damage to tissues than the performic acid-Schiff method given below.

THE PERFORMIC ACID-SCHIFF METHOD FOR LIPIDS CONTAINING UNSATURATED BONDS (Lillie, 1952)

(*Formalin, Helly, Zenker; paraffin sections. Formalin; frozen sections*)

Preparation of Solutions

(1) **Performic Acid (Pearse).** This solution is that given in the oxidation technique for disulphide-containing proteins in Appendix 5.

(2) **Performic Acid (Lillie).** To 9·2 g. 90 per cent. formic acid (8 ml.) add 33·7 g. 30 per cent. H_2O_2 (31 ml.) and 0·4 g. conc. H_2SO_4 (0·22 ml.). Keep temperature below 25°. 4·7 per cent. performic acid is formed within 2 hours and the solution deteriorates after 2 hours.

(3) **Peracetic Acid.** This reagent (40 per cent.) is available commercially. A similar solution may be made (Pearse, 1951) by adding 20 ml. of acetic anhydride to 5 ml. of 30 per cent. H_2O_2. After thorough mixing, and standing for 24 hours, the solution is diluted to twice its volume before use.

(4) **Schiff's Reagent.** Any except the thionyl chloride modification may be used.

Method

(1) Bring sections to water, removing mercury precipitate if necessary.
(2) Blot dry.
(3) Treat with performic or peracetic acids for 1–2 hours.
(4) Wash in running water for 10 minutes.
(5) Immerse in Schiff's solution for 10 minutes.
(6) Wash in 3 changes of sulphite water for 2 minutes each.
(7) Wash in warm running water for 10 minutes.
(8) Mount in glycerine jelly without counterstaining.

Lillie recommends counterstaining in Weigert's acid hæmatoxylin and in saturated aqueous picric acid, dehydration in alcohol, clearing in xylene and mounting in a synthetic medium. He particularly recommends the nuclear counterstain for covering up the Feulgen-stained nuclei. This practice is not recommendable histochemically. The increased basophilia of the tissues after performic acid oxidation (see Chapter V, p. 111) causes basic dyes to overlay a number of structures in which a positive PFAS reaction is visible. The red compound formed in the tissues is partially soluble in alcohol and dehydration is therefore best avoided.

Result

Structures which stain red may be regarded as containing lipids (phospholipid or cerebroside) with unsaturated bonds.

Lillie regards the reaction as specific for the ethylene group HC = CH and recommends controlling this point by means of control sections treated with bromine (directions on p. 846). The ethylene groups are blocked by this procedure and the PFAS reaction becomes negative. The PAS reaction, however, where it is due to the 1 : 2-glycol group, remains positive and is reversible by acetylation (Appendix 9, p. 832).

METHODS FOR CHOLESTEROL AND ITS ESTERS

Schultz Method (after Weber *et al.*)

(Cold calcium-cadmium-formalin; frozen sections)

Method

(1) Cut frozen sections at 20–30 μ.
(2) Wash for 24 hours in distilled water (several changes).
(3) Treat with 2·5 per cent. ferric ammonium sulphate in 0·2 M-acetate buffer at 37° for 7 days. It is recommended that the buffer should be adjusted to pH 3 by mixing 2 ml. of 0·2 M-sodium acetate with 98 ml. of 0·2 M-acetic acid. The final pH is about 2.
(4) Wash sections for 1 hour each in 3 changes of acetate buffer.

(5) Rinse in distilled water, transfer to 5 per cent. formalin for 10 minutes.

(6) Mount sections on slides and remove excess water by blotting the edges. Do not dry the sections.

(7) Place one drop of a mixture of equal parts of sulphuric and acetic acids on a coverslip. Invert the slide and apply it to the coverslip. Turn right way up and apply even pressure to the coverslip so as to flatten the section. Grasp the coverslip at the corners and oscillate several times.

Result

Cholesterol and cholesterol esters appear pale violet or red, turning rapidly to green within a few seconds. The colour remains stable for 30–60 minutes.

DIFFERENTIATION OF CHOLESTEROL FROM ITS ESTERS (after Feigin, 1956)

Method

(1) Immerse formalin-fixed frozen sections in a 0·5 per cent. solution of digitonin in 40 per cent. ethanol for 3 hours at room temperature.

(2) Drain and immerse in a mixture of equal parts of absolute ethanol and ether for 3 hours at room temperature.

(3) Drain and immerse, together with an untreated control section, in 2·5 per cent. aqueous iron alum at 37° for 2–4 days.

(4) Drain dry and carry out stage 7 of the Schultz method.

Result

Cholesterol esters are extracted by the alcohol-ether mixture and therefore stain only in the control section. The free cholesterol-digitonin complex is not extracted and therefore stains in both test and control sections.

Okamoto Method (after Ueda)

(*Formalin; frozen sections*)

Method

(1) Mount the section on a slide and blot thoroughly.

(2) Cover with 2 drops of freshly made sulphuric-iodine (conc. H_2SO_4, 10–12 ml., alcoholic iodine,* 20 ml.).

(3) Apply a coverslip and examine under the microscope.

Result

Cholesterol and its esters give a blue-green colour stable at most for 2–3 hours.

Bismuth Trichloride Method (Grundland, Bulliard and Maillet, 1949)

Preparation of the Reagent

Bismuth trichloride . . .	0·2 g.
Acetyl chloride	1·0 ml.
Anhydrous nitrobenzene. . .	100·0 ml.

* Iodine 6·5 g.
KI 2·5 g.
95 per cent. alcohol 180 ml.

Method

(1) Fix small pieces of tissue for 24–48 hours in 70 per cent. alcohol saturated with digitonin.

(2) Allow the alcohol to evaporate and immerse in molten paraffin wax containing 5 per cent. glyceryl monostearate for 12 hours.

(3) Embed in paraffin.

(4) Cut sections (6–10 μ) and mount on slides. Do not remove wax.

(5) Expose to the bismuth trichloride reagent for 15–45 minutes.

(6) Rinse rapidly in 10 per cent. acetyl chloride in nitrobenzene.

(7) Clear and remove wax in benzene.

(8) Mount in liquid paraffin.

Result

Cholesterol appears brown, its esters are not revealed. (The preparations are stable for a long period.)

Further treatment can be employed to accentuate the colour of the reaction. For this purpose proceed thus after stage 6 :

(1) Wash in absolute alcohol/conc. nitric acid (1 : 3) to remove excess bismuth.

(2) Wash rapidly in absolute alcohol.

(3) Immerse in a dilute solution of yellow ammonium sulphide.

(4) Wash in absolute alcohol.

(5) Clear in xylene and mount in Canada balsam.

MODIFIED HALE METHOD SUITABLE FOR NERVOUS TISSUES (Seitelberger, Vogel and Stepan, 1957)

Method

(1) Mount formalin-fixed frozen sections on slides. Bring these, or paraffin sections to 50 per cent. alcohol.

(2) Stain for 10 minutes in Sudan IV in Herxheimer's solution.

(3) Pass rapidly through 70 per cent. alcohol and wash twice in distilled water.

(4) Transfer for 2 minutes to the colloidal iron solution (see p. 836).

(5) Wash in distilled water.

(6) Treat for 10 minutes with acid ferrocyanide (1 per cent. HCl, 2 parts; 2 per cent. potassium ferrocyanide, 1 part).

(7) Wash twice in distilled water.

(8) Fix in alum solution for ten minutes. (Aluminium ammonium sulphate.24 H_2O, 26 g. ; 28 per cent. ammonia 5 ml. ; distilled water 450 ml.).

(9) Wash in distilled water.

(10) Mount in glycerine jelly.

Result (Plate IIIA)

Acidic polysaccharides, free or bound, stain deep blue. Neutral fats red.

APPENDIX 12

REACTIONS FOR ALDEHYDES

The method of using Schiff's reagent for this purpose does not differ from its use in the Feulgen reaction, already described in Chapter VIII and in Appendix 8, p. 822.

Ammoniacal Silver Reaction

Gomori's hexamine-silver solution is recommended.

Preparation of the Solution

Add 5 ml. 5 per cent. silver nitrate to 100 ml. 3 per cent. aqueous hexamine. A precipitate forms which redissolves. Add 5 ml. of borate buffer (approximately pH 8) made by adding a drop of alcoholic phenolphthalein to 3 per cent. boric acid and then adding N-NaOH until a pink colour appears. Finally, make up to 200 ml. with distilled water.

Method

(1) Bring paraffin sections to water.
(2) Oxidize sections (for instance with 5 per cent. chromic acid for 1 hour, or with 1 per cent. periodic acid for 5 minutes).
(3) Wash in running water for 10 minutes.
(4) Immerse in the hexamine-silver solution for 1–3 hours at 37°.
(5) Wash in distilled water.
(6) Rinse in 5 per cent. sodium thiosulphate, 30 seconds.
(7) Wash and counterstain nuclei, if desired.

Result (Fig. 93)

Aldehyde groups are revealed by a brown or black deposit of silver.

The Naphthoic Acid Hydrazide Reaction (after Ashbel and Seligman)

The synthesis of this reagent is carried out according to details given in Appendix 8, p. 823. It can be applied to tissues fixed in any kind of fixative, and to frozen or paraffin sections, after the application of any reagent known to produce aldehyde groups. Besides these the reaction is given by sulphonic and sulphinic acids (see Chapter V, p. 112) and by ketones if these are present in sufficient amounts.

Preparation of Solutions

(1) 0·1 per cent. 2-hydroxy-3-naphthoic acid hydrazide in 50 per cent. reagent ethanol containing 5 per cent. acetic acid. Solution is effected by warming to 70° and shaking vigorously.
(2) Alcoholic phosphate buffer (pH 7·2–7·5) prepared by mixing equal quantities of 0·1 M buffer and absolute reagent ethanol.

Method

(1) If frozen sections of formalin-fixed tissues are used, formaldehyde may still be present. This may be removed either by thorough washing or,

preferably, by blocking treatment with hydroxylamine (p. 865). In the case of paraffin sections such treatment is unnecessary.

(2) Immerse sections in the fresh hydrazide solution at 60° for 1–3 hours.

(3) Wash in three changes of 50 per cent. ethanol with 5 per cent. acetic acid.

(4) Wash in three changes of 50 per cent. ethanol.

(5) Wash in distilled water.

(6) Transfer to the alcoholic buffer solution in a Coplin jar.

(7) Add either 5–10 ml. of freshly diazotized *o*-dianisidine or 50 mg. Fast Blue B salt (I.C.I.) in 0·5 ml. distilled water. Agitate the sections to effect mixture. Leave the sections for 2–5 minutes.

(8) Wash in running water.

(9) Mount frozen sections in glycerine jelly, paraffin sections in DPX or Canada balsam after rapid dehydration in alcohol and clearing in xylene.

Result (Figs. 65 and 98)

Tissue components in which aldehydes are present, or in which they have been produced, bluish to reddish-purple. Various cytoplasmic proteins and lipids may be coloured pink or red, only blue shades constitute a positive reaction.

The Phenylhydrazine-formazan Reaction

(*Formalin; frozen or paraffin sections*)

This reaction has been used to demonstrate tissue aldehydes formed by periodic acid oxidation. It can also be used, as shown by Karnovsky and Deane (1955) to demonstrate carbonyls (aldehyde) formed during autoxidation of tissue lipids. For this purpose, of course, stages 2 and 3 are unnecessary.

Solutions

(1)	Phenylhydrazine	5 ml.
	Glacial acetic acid	10 ml.
	Distilled water	30 ml.
(2)	Pyridine redistilled	30 ml.
	Distilled water	10 ml.

To solution 2 add 100 mg. of the stable diazotate of *o*-dianisidine (Fast Blue B salt) or 5-nitroanisidine (Fast Red R salt) just before use.

Method

(1) Bring paraffin sections to water ; mount frozen sections on slides after thorough washing to remove formalin.

(2) Oxidize for 5–10 minutes with 1 per cent. aqueous periodic acid.

(3) Wash in running water.

(4) Treat with the acid phenylhydrazine solution for 12–24 hours at 22° or, preferably, for 30 minutes at 60°.

(5) Wash in two changes of 5 per cent. acetic acid.

(6) Wash in water.

(7) Treat with the diazonium salt in pyridine for 4–6 minutes.

(8) Wash quickly in acetone and then in water.

(9) Dehydrate quickly in alcohol, clear in xylene and mount in Canada balsam.

Result (Fig. 95)

Structures containing aldehyde groups may give a positive reaction, appearing red or reddish-brown.

BLOCKING REAGENTS FOR ALDEHYDES AND KETONES

(*various*)

(1) Bisulphite

Add 10 ml. ethyl alcohol to 40 ml. 50 per cent. aqueous sodium bisulphite. Expose tissues for 2–8 hours at room temperature.

(2) Phenylhydrazine

Phenylhydrazine	5 ml.
Glacial acetic acid	10 ml.
Distilled water	to 50 ml.

Expose tissues for 2–3 hours at 60°.

(3) 2 : 4-Dinitrophenylhydrazine

A saturated solution in N-HCl at 0°. Expose tissues for 20 hours at 0°, wash in cold N-HCl and then in water.

(4) Semicarbazide

Semicarbazide hydrochloride . .	2 g.
Sodium acetate (crystalline) . .	5 g.
Distilled water	40 ml.

Expose tissues for 2–3 hours at 60°.

(5) Thiosemicarbazide

Thiosemicarbazide	4 g.
Glacial acetic acid	2 ml.
Distilled water	40 ml.

Expose tissues for 2–3 hours at 60°.

(6) Hydroxylamine

Hydroxylamine hydrochloride. .	10 g.
Sodium acetate (crystalline) . .	20 g.
Distilled water	40 ml.

Expose tissues for 1–3 hours at 22°.

(7) Amine-aldehyde Condensation

Sulphanilic acid	50 mg.
0·1 M-KH_2PO_4	40 ml.

Incubate sections for 24 hours at 37°.

(8) Amine-aldehyde Condensation (Lillie, 1954)

Aniline	9 ml.
Conc. HCl	8 ml.

Shake during addition and dilute with

Distilled water	100 ml.

Incubate sections for 1–6 hours at 22°.

Amine-aldehyde Condensation (Lillie and Glenner, 1957)

Aniline	10 ml.
Acetic acid	90 ml.

Incubate sections for 20–30 minutes at 22°.

(9) Dimedone (Fig. 96)

A saturated solution in 5 per cent. acetic acid. Treat sections for 1–16 hours at 60° or for 2–3 days at 22°.

(10) Cannizzaro Reaction

Treat blocks of tissue with reagents necessary to produce aldehyde groups (HCl, CrO_3, HIO_4, etc.). Treat with 50 per cent. KOH in 50 per cent. ethanol for 1–4 hours, and wash thoroughly in water.

(11) Meerwein-Ponndorf Reaction

Treat blocks of tissue as for the Cannizzaro reaction to produce aldehydes. Heat for 2–4 hours under a reflux condenser in isopropanol containing excess aluminium isopropoxide.

THE PLASMAL REACTION (after Hayes, 1949)

(*unfixed, cold microtome sections or smears*)

Method

(1) Cut frozen sections 10–15 μ. Mount on coverslips or slides as desired.
(2) Wash in several changes of distilled water.
(3) Place one section in 1 per cent. aqueous $HgCl_2$ for 2–10 minutes.
(4) Place this section and an untreated control section in Schiff's reagent for 5–15 minutes.
(5) Wash in three changes of bisulphite water (5 ml. 10 per cent. $K_2S_2O_5$, 5 ml. N-HCl, 100 ml. distilled water), 2 minutes in each change.
(6) Wash in water.
(7) Mount in glycerine jelly, or in DPX, after mounting the section on a slide, dehydrating in gradual alcohols and clearing in xylene.
(Glass section lifters must be used throughout.)

Result

Acetal lipids, reddish-purple.

THE PLASMAL REACTION (after Cain, 1949)

(*fresh tissues*)

Method

(1) Drop pieces of fresh tissue into a mixture of equal parts of (A) Schiff's solution half diluted with bisulphite water and (B) saturated (7 per cent.) aqueous $HgCl_2$. Leave for 15 minutes.
(2) Wash in several changes of bisulphite water.
(3) Treat with 10 per cent. formalin for 2 hours.
(4) Wash in running water for 2 hours or until control pieces become pale lilac.
(5) Cut frozen sections.
(6) Mount on slides and enclose in glycerine jelly or dehydrate, clear and mount in DPX.

Cain used control sections stained with Sudan black B to indicate that the plasmal formed was derived from lipid sources.

Result

Acetal lipids, reddish-purple.

PHOSPHOLIPIDS BY THE PSEUDO-PLASMAL REACTION

(Cold formalin; frozen sections)

The plasmal reaction may be used, on frozen sections of tissues fixed in formalin for 4–16 hours at 4°, as an indication of the presence of associated phospholipids.

Method

(1) Treat a number of sections with hydroxylamine (see Blocking Reagents, above) for 1–3 hours at 22°.
(2) Wash the sections in three changes of distilled water.
(3) Treat some of the sections with 1 per cent. $HgCl_2$ for 10 minutes.
(4) Wash in distilled water.
(5) Immerse treated sections, and untreated control sections, in Schiff's reagent for 15–20 minutes.
(6) Wash in bisulphite water, 10 minutes.
(7) Wash in water.
(8) Mount in glycerine jelly.

Result

Control hydroxylamine-treated sections unexposed to $HgCl_2$ should be absolutely colourless. Lipids containing acetals and easily-revealed aldehyde groups appear reddish-purple in sections treated with $HgCl_2$.

METHOD FOR α-KETOL GROUPS OF CORTICOIDS

(After Khanolkar *et al.*, 1958)

(Fresh, cold microtome, sections ; mounted on slides or coverslips)

This method is based on the oxidation of α-ketols to keto-aldehydes by ferric chloride. Its theoretical specificity is high and *in vitro* tests support the author's views.

Method

(1) Block existing aldehydes by immersing sections in Lillie's acetic-aniline (p. 866) for 20 minutes at room temperature.
(2) Rinse gently in distilled water for 1–3 minutes.
(3) Incubate in 5 per cent. ferric chloride at 50° for 30 minutes.
(4) Rinse gently in distilled water.
(5) Immerse in Schiff's reagent for 20 minutes.
(6) Wash in 3 changes of bisulphite water (see above).
(7) Mount in glycerine jelly.

Result

Corticoids are revealed by magenta staining.
Androgens, œstrogens, pregnandiol and cholesterol do not react.

APPENDIX 14

THE CALCIUM-COBALT METHOD FOR ALKALINE PHOSPHATASE (after Gomori)

(Cold acetone, paraffin sections; cold formalin, frozen sections)

Paraffin Sections

THIN blocks of tissue should be fixed, according to Gomori's (1946) method, in two or three changes of cold absolute acetone at 4° for 24 hours. There are many subsequent ways in which the tissues may be embedded in paraffin wax, most of them being minor variations designed to minimize destruction of the enzyme. As a routine procedure the following method gives good results.

(1) Transfer the blocks progressively at ½-hourly intervals to absolute ethanol, absolute ethanol-ether with one or two changes, and thence to 1 per cent. celloidin.

(2) Drain off excess celloidin and harden in chloroform.

(3) Clear in benzene.

(4) Embed in paraffin wax, avoiding prolonged exposure to the high temperature of the wax bath.

(5) Cut sections at 5 μ and mount on albuminized slides.

(6) Dry the slides for 3 hours at 37°.

(7) Store at 4° until required for incubation.

Method Proper

(1) Remove wax from the slides by brief immersion in light petroleum.

(2) Pass to water via absolute acetone.

(3) Incubate for ½–16 hours, at 37°, in the following medium :

10 ml. 3 per cent. sodium β-glycerophosphate.
10 ml. 2 per cent. sodium diethyl barbiturate.
5 ml. distilled water.
20 ml. 2 per cent. calcium chloride.
1 ml. 5 per cent. magnesium sulphate.

(4) Rinse in running water.

(5) Treat with 2 per cent. cobalt nitrate or acetate, 3–5 minutes.

(6) Rinse well in distilled water.

(7) Treat with a dilute solution of yellow ammonium sulphide, 1–2 minutes.

(8) Wash in water, counterstain in 1 per cent. eosin, 5 minutes, if desired.

(9) Dehydrate in alcohol, clear in xylene and mount in Canada balsam.

Result (Figs. 114 and 116)

Various structures are stained black or brownish-black in tissues possessing alkaline phosphatase activity.

Frozen Sections

Method

(1) Cut sections 10–15 μ thick and mount on clean glass slides without any adhesive.

(2) Dry in air at room temperature for 1–2 hours.
(3) Incubate in the substrate solution for ½–4 hours.
(4) Wash in water, treat with 2 per cent. cobalt solutions, wash, treat with dilute yellow ammonium sulphide.
(5) Counterstain in 1 per cent. aqueous eosin, 5 minutes.
(6) Wash in running water, 5 minutes.
(7) Mount in glycerine jelly.

MODIFIED GOMORI METHOD FOR ALKALINE PHOSPHATASE (after Fredricsson, 1952, 1956)

(*Alcohol, paraffin sections*)

Method

(1) Fix small blocks of tissue in 90 per cent. alcohol for 24 hours at 22° (two changes).
(2) Transfer to 96 per cent. alcohol for 1 hour.
(3) Transfer to two changes of absolute alcohol, 15 minutes each.
(4) Two changes in benzene, 30 minutes each.
(5) Embed in paraffin wax at 56° (3 changes, 20 minutes each).
(6) Cut sections 3–10 μ and mount on slides without floating out on water.
(7) Remove wax with xylene and pass through absolute acetone, and 40 per cent. acetone, to water.
(8) Incubate for 10–60 minutes in a closed Coplin jar at 37° in the following mixture :

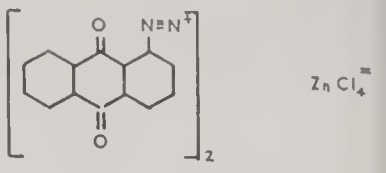
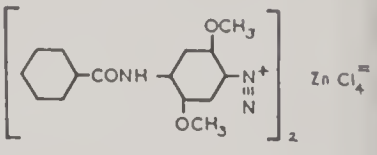
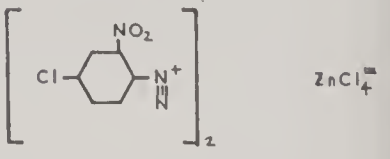
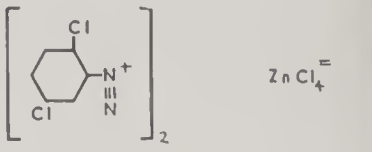
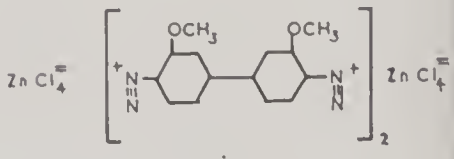
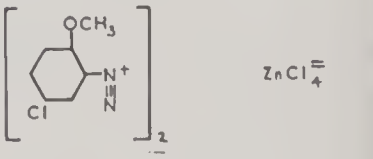
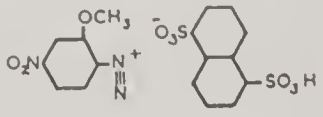
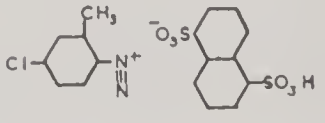
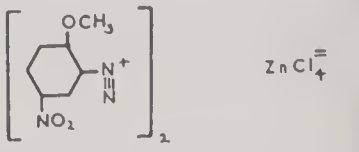
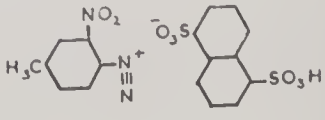
2 per cent. sodium-β-glycerophosphate .	25 ml.
2 per cent. sodium veronal . . .	25 ml.
2 per cent. calcium nitrate . . .	5 ml.
0·8 per cent. magnesium chloride . .	5 ml.
Acetone	40 ml.

(9) Rinse in 40 per cent. acetone.
(10) Treat with 2 per cent. cobalt nitrate in 40 per cent. acetone for 5 minutes.
(11) Rinse in 40 per cent. acetone.
(12) Treat with dilute yellow ammonium sulphide in 40 per cent. acetone for 3 minutes.
(13) Rinse in acetone, continue dehydration in alcohol.
(14) Clear in xylene and mount in Canada balsam.

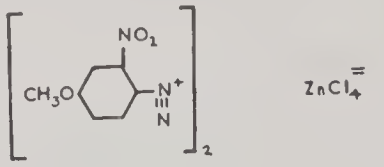
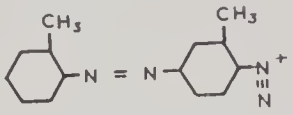
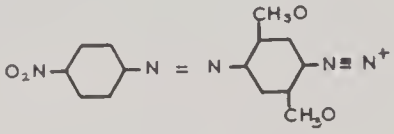
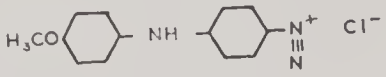
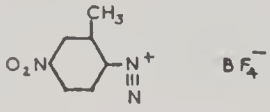
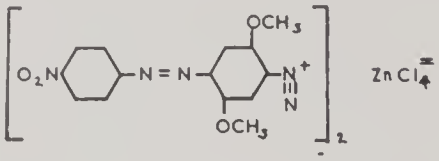
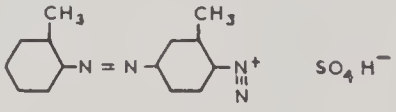
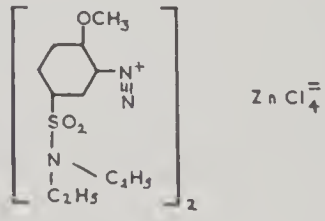
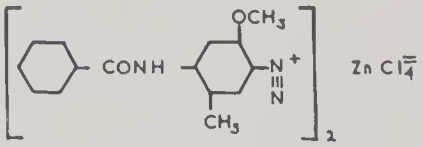
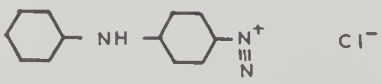
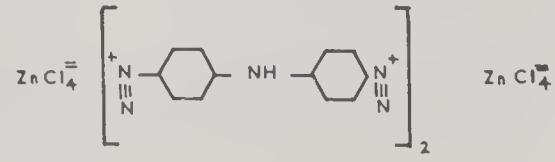
Result

Clean black deposits of cobalt sulphide indicate sites of enzyme activity.

Diazonium Salts in Enzyme Histochemistry

(No.)	Salt Name	Chemical Composition	Formula (Stabiliser may vary)
1	Fast Red AL	1-amino-anthraquinone	
2	Fast Blue RR (Echtblausalz RR)	4-benzoylamino-2 : 5-dimethoxyaniline	
3	Fast Red GG	*p*-nitroaniline	
4	Fast Red 3G (Echtrotsalz 3GL)	4-chloro-2-nitroaniline	
5	Fast Scarlet GG	2 : 5-dichloroaniline	
6	Fast Blue B (Echtblausalz B)	*o*-dianisidine	
7	Fast Red RC (Echtrotsalz RC)	4-chloro-*o*-anisidine	
8	Fast Red B (Echtrotsalz B)	5-nitro-*o*-anisidine	
9	Fast Red TR (Echtrotsalz TR)	5-chloro-*o*-toluidine	
10	Fast Scarlet R	4-nitro-*o*-anisidine	
11	Fast Red G (Echtrotsalz 3 GL)	3-nitro-*p*-toluidine	

DIAZONIUM SALTS IN ENZYME HISTOCHEMISTRY—*continued*

(No.)	Salt Name	Chemical Composition	Formula (Stabilizer may vary)
12	Bordeaux GP	3-nitro-*p*-anisidine	
13	—	4-amino-3 : 1′-dimethyl azobenzene	
14	Echtschwarzsalz K	4-amino-2 : 5-dimethoxy 4′-nitro azobenzene	
15	Variamine Blue B (Variaminblausalz B)	4-amino-4′-methoxy diphenylamine	
16	Fast Red RL	3-nitro-*o*-toluidine	
17	Fast Black K	4-amino-4′-nitro-3 : 6-dimethoxy-azobenzene	
18	Fast Garnet GBC (Echtgranatsalz GBC)	4-amino-3 : 1′-dimethyl azobenzene	
19	Fast Red ITR (Echtrotsalz ITR)	2-methoxy-5-diethylamino sulphaniline	
20	Fast Violet B (Echtvioletsalz B)	2-benzoylamino-4-methoxy toluidine	
21	Fast Blue RT (Variaminblausalz B)	4-amino-diphenylamine	
22	Fast Black B (Echtschwarz B)	*p*, *p*′-diaminodiphenylamine	

A MODIFIED COUPLING AZO DYE METHOD FOR ALKALINE PHOSPHATASE

(*Cold formalin or fresh frozen sections; freeze-dried paraffin sections*)

Frozen Sections

Method

(1) Fix thin slices of tissues in 10 per cent. neutral formalin at 4° for 10–16 hours. Alternatively use fresh frozen cold microtome sections, mounted on cover slips.

(2) Cut frozen sections 10–15 μ thick and mount on clean slides without adhesive.

(3) Allow to dry in air for 1–3 hours to ensure adherence.

(4) Dissolve 10–20 mg. sodium α-naphthyl phosphate in 20 ml. 0·1 M stock " tris " buffer (pH 10).* Add 20 mg. of the stable diazotate of 5-chloro-*o*-toluidine (Salt 9, Table 30, p. 407) and stir well. Filter on to the slides sufficient to cover each section adequately and incubate at room temperature (17°–22°) † for 15–60 minutes.‡ (Alternatively, use the same quantity of the stable diazotate of 2-benzoylamino-4-methoxy toluidine (Salt 20, Table 30), or of *p-p'*-diaminodiphenylamine (Salt 22, Table 30), and proceed in the same manner.)

(5) Wash in running water for 1–3 minutes.

(6) Counterstain in Mayer's hæmalum, 4–6 minutes.

(7) Wash in running water for 30–60 minutes.

(8) Mount in glycerine jelly.

Result (Plate IVA)

The sites of alkaline phosphatase activity are coloured brown with Salts 9 and 20 or black with Salt 22. Nuclei, dark blue.

Paraffin Sections

(*Cold acetone-fixed, paraffin-embedded*)

Method

(1) Bring sections to water via absolute acetone after removing the paraffin with light petroleum.

(2) Cover with freshly made and filtered substrate-diazonium salt mixture as above.

(3) Incubate for 30 minutes to 4 hours (Salt 2) or for up to 2 hours (Salt 7) or for up to 12 hours (Salt 9).

(4) Wash in water, counterstain as above and blue in running water.

(5) Mount in glycerine jelly.

(The use of Salt 9 is particularly recommended.)

Result (Figs. 105 and 111)

With Salt 9, the sites of alkaline phosphatase activity appear dark reddish-brown, localization is excellent. Nuclei, blue.

* The pH of the medium is lowered both by the addition of the substrate and also by the various diazotates.

† With temperatures above 22°, diffusion and abnormal crystallization effects may be noted.

‡ It is possible to remove the aluminium sulphate, present in most stable diazotates, by the addition of a few ml. of a saturated aqueous solution of purpurin. The red aluminium lake is retained by a Whatman No. 40 paper. This procedure appreciably increases the speed of the reaction. Removal of inhibitory zinc salts by means of chelators such as dithizone unfortunately removes the diazotates also.

AZO DYE METHOD FOR LEUCOCYTE PHOSPHATASE
(after Kaplow, Monis, Hayhoe)

(Formalin-methanol)

Method

(1) Prepare smears from capillary or sequestrene-venous blood.
(2) Allow the freshly made smears to dry in air for a few minutes.
(3) Fix for 30 seconds in ice-cold 10 per cent. formalin in methanol (40 per cent. formaldehyde, 10 ml. ; methanol, 90 ml.).
(4) Prepare incubating solution A* or B† and filter directly on to the smears. Incubate 5–10 minutes at 22° (A) or 30 minutes (B).
(5) Wash in running water.
(6) Counterstain with Mayer's hæmalum for 1½–3 minutes or with 2 per cent. chloroform-washed methyl green for 2 minutes.
(7) Wash in running water.
(8) Mount in glycerine jelly.

Result

Neutrophil granulocytes (40–90 per cent.) show alkaline phosphatase activity as a brown, red or blue deposit.

PREPARATION OF SODIUM α-NAPHTHYL ACID PHOSPHATE
(Friedman and Seligman, 1950)

(1) α-naphthylphosphoryl dichloride

Heat 25 g. α-naphthol and 26·5 g. (15·5 ml.) phosphorus oxychloride in 90 ml. dry benzene under a reflux condenser and add 13·7 g. dry pyridine over a period of 30 minutes. Heat for a further 15 minutes, cool and remove precipitate of pyridine hydrochloride by filtration. Distil off the solvent and redistil to leave a clear colourless syrup (b.p. 199°–201°).

(½) α-naphthyl acid phosphate

Place a thin layer of α-naphthylphosphoryl dichloride over a layer of aqueous KOH in a Petri dish in a partially evacuated desiccator. Quantitative conversion to α-naphthyl phosphate occurs with formation of a white crystalline solid (m.p. 155°–157°). The monosodium salt is precipitated from a solution of the latter in methanol by the addition of an equivalent of sodium methoxide.

PREPARATION OF NAPHTHYL AS-PHOSPHATES
(Burstone, 1958)

A suspension of 5 gm. of the appropriate hydroxy-naphthoic arylide (Naphthols AS, AS-MX, AS-TR, AS-D, AS-CL, etc.) in 50–70 ml. of tetrahydrofuran is treated with excess $POCl_3$ (4 ml.) in the presence of sodium chloride (1 gm.), sodium sulphate (1 gm.) and tetrasodium ethylene diamine tetra-acetic acid (150 mg.). Pyridine (2 ml.) is then added to the mixture

* To 25 ml. 0·1 M-" tris " buffer (pH 9·2) add 20 mg. sodium α-naphthyl and phosphate and 30 mg. Fast Garnet GBC salt (No. 18).
† To 5 ml. " Stock " Naphthol AS-MX phosphate add 10 mg. Fast violet LB salt or Fast blue RR salt (No.2).

with constant stirring. After standing for 18 hours the acid phosphate is prepared by evaporating the solvent in a stream of air and adding to the resulting slush one or two cubes of ice. The reaction mixture is then added to 500 ml. cold water, stirred, and collected under suction.

A stock solution ready for histochemical use can be prepared from the majority of AS acid phosphates by dissolving 50 mg. in 20 ml. N,N'-dimethyl formamide, adding 20 ml. water and sufficient molar Na_2CO_3 to bring the pH to 8·0. After the addition of 600 ml. water the volume is brought up to 1 litre by adding 0·2 M-" tris " buffer (pH 8·3). The opalescent solution is stable at room temperature for several months. The disodium salts of the AS phosphates are prepared, if required, by treatment with sodium methylate in methanol or with sodium carbonate.

NAPHTHOL AS-PHOSPHATE AZO DYE METHOD
(after Burstone)

(*Freeze-dried or cold acetone-fixed; paraffin sections*)

Method

(1) Bring sections to water via xylene and acetone.

(2) Incubate for 5–30 minutes at 22° in 40 ml. Stock Solution * (*vide supra*) containing 40 mg. of the chosen diazonium salt (Fast red-violet LB salt, Fast blue RR, red RC and red TR are recommended).

(3) Wash briefly in water and mount in PVP medium (Burstone, 1957).†

Result

Intensely coloured red or blue azo dyes indicate sites of alkaline phosphatase activity.

CALCIUM METHOD FOR 5-NUCLEOTIDASE
(after Pearse and Reis)

(*Cold acetone, double embedded sections; cold formalin, frozen sections*)

Method

(1) Prepare acetone-fixed paraffin sections or formalin-fixed frozen sections as for the methods described for alkaline phosphatase.

(2) Incubate one section (with glycerophosphate or phenyl phosphate) as for alkaline phosphatase at pH 9·2, another similarly at pH 7·5, a third in the following medium at pH 7·5 and a fourth in the same medium containing water instead of substrate, all for 3–18 hours at 37°.

5 vol. barbiturate buffer (pH 7·5)
(sodium diethyl barbiturate, 0·1 M, 3 vol. ; HCl, 0·1 N, 2 vol. ; distilled water, 1 vol.).
1 vol. 12 per cent. (w/v) $Ca(NO_3)_2$.
1 vol. 2 per cent. (w/v) $MgCl_3$.
1 vol. 0·04 M-adenylic acid (adenosine-5-phosphate).

(3) Wash with 2 per cent. $Ca(NO_3)_2$ of pH about 8·0 and then in distilled water.

(4) Immerse in 1 per cent. $AgNO_3$ and expose to daylight for 1 hour.

* According to Burstone AS-D, AS-OL, AS-BS and AS-MX phosphates are rapidly hydrolysed at alkaline pH levels.

† Dissolve 50 gm. polyvinyl pyrrolidone in 50 ml. distilled water. Stand overnight. Add 2 ml. glycerol and a crystal of thymol and stir. (Refractive Index 1·43, increasing as evaporation of water takes place.)

(5) Rinse in distilled water and treat with 5 per cent. $Na_2S_2O_3$ for 10 minutes.

(6) Wash, dehydrate in alcohol, clear in xylene and mount in Canada balsam.

The Co and $(NH_4)_2S_2$ method for revealing the precipitate of calcium phosphate may be used as an alternative.

Result (Figs. 115 and 117)

Various structures in the neighbourhood of weak or moderately strong sources of 5-nucleotidase are stained black. In the case of very strong sources of enzyme, localization to a group of cells may be possible. (The glycerophosphate control at pH 7·5 must be subtracted from the total result given by adenylic acid at pH 7·5 to obtain a true estimate of 5-nucleotidase. This control is often negative and the substrate control must be negative.)

LEAD METHOD FOR 5-NUCLEOTIDASE (after Wachstein and Meisel)

(*Free-floating fresh frozen sections*)

Method

(1) Cut 10–15 μ sections from the cold microtome directly into the following medium :

10 ml. of 1·25 per cent. adenosine-5-phosphate.
5 ml. of 0·2 M-" tris " buffer at pH 7·2.
30 ml. of 0·2 per cent. $Pb(NO_3)_2$.
5 ml. of 0·1 M-$MgSO_4$.

(2) Remove from cryostat and incubate sections for $\frac{1}{2}$–2 hours at 37°. (Immerse any floating sections with the aid of a glass needle.)

(3) Stop reaction with 2 ml. of 40 per cent. formaldehyde, 30 minutes.

(3) Remove sections with section lifter and rinse in distilled water.

(4) Treat with dilute yellow ammonium sulphide, 2 minutes.

(5) Rinse in distilled water.

(6) Mount on slides and allow to dry (almost).

(7) Apply glycerine jelly and coverslip.

Result

Brown deposits of lead sulphide indicate sites of enzyme activity.

A CALCIUM-COBALT METHOD FOR ALDOLASE (after Allen and Bourne)

(*Alcohol, frozen sections*)

The authors used tissues fixed in 80 per cent. alcohol for 24 hours, from which frozen sections were cut after removal of the alcohol by washing in water.

Preparation of Solutions

(1) The Precipitant Mixture. Dissolve 5·5 g. $MgCl_2.6H_2O$ and 7·0 g. NH_4Cl in 35 ml. 5N NH_4OH. Stand for 1 hour, filter and add 60 ml. 4N NH_4OH to the filtrate.

(2) Purified Substrate Solutions A and B. (*A*) Mix 40 ml. 4 per cent. sodium hexose diphosphate with 20 ml. of the precipitant mixture. Stand for 30 minutes and filter.

(*B*) Mix 20 ml. of 4 per cent. sodium hexose diphosphate with 20 ml. of the precipitant mixture and 20 ml. of distilled water. Stand for 30 minutes and filter.

(3) **Final Substrate Solutions A and B.** (*A*) Add 10 ml. purified substrate solution A to 1·7 ml. of 0·1 M-sodium iodoacetate and 5 ml. distilled water.

(*B*) Add 15 ml. purified substrate solution B to 2·5 ml. of 0·1 M-sodium iodoacetate and 7·5 ml. distilled water.

Method

(1) Mount frozen sections on clean slides without adhesive and dry at room temperature for 1–3 hours.

(2) Incubate sections in final substrate solutions A and B for 1–2 hours at 37°.

(3) Immerse in 2 per cent. cobalt acetate for 5–10 minutes.

(4) Wash and immerse in dilute yellow ammonium sulphide solution 1–2 minutes.

(5) Dehydrate in alcohol, clear in xylene, mount in Canada balsam.

Result

A brownish-black precipitate in either section indicates the presence in the section of aldolase.

CALCIUM METHOD FOR ADENOSINE TRIPHOSPHATASE
(after Maengwyn-Davies *et al.*)

(Unfixed frozen sections)

Preparation of Incubating Medium

This must be prepared immediately before use, heated to 37° for 1 hour, filtered, and finally adjusted to pH 9·9 with molar KOH.

0·1 M-glycine 0·4 M-KCl } in saturated sodium acetate * .	12 ml.
Saturated sodium acetate	12 ml.
0·36 M-$CaCl_2$	3·6 ml.
M-KOH	0·6 ml.
0·04 M-sodium adenosine triphosphate † . .	6 ml.
Distilled water	13·8 ml.
Saturated sodium phosphate ($NaPO_4$) . .	0·3 ml.

Method

(1) Incubate free-floating sections (10–15 μ) for 5 minutes to 3 hours.
(2) Wash in three changes of 1 per cent. $CaCl_2$ in 75 per cent. ethanol.
(3) Transfer to 2 per cent. $CoCl_2$ for 3 minutes.
(4) Develop in 1 per cent. yellow ammonium sulphide.
(5) Wash, dehydrate and mount in a suitable synthetic medium.

Result

ATPase activity shows as a blackish-brown deposit.

* Made up by dissolving the glycine and KCl in a small amount of water and adding saturated sodium acetate to make up the volume.

† Final concentration, 0·005 M.

CALCIUM METHOD FOR ADENOSINE TRIPHOSPHATASE
(after Padykula and Herman)

(Unfixed frozen sections; free-floating or attached to cover slips)

Preparation of Incubating Medium

This solution must be absolutely freshly prepared.

0·1 M-sodium barbiturate (2·062 g./100 ml.)	20 ml.
0·18 M-$CaCl_2$ (1·998 g./100 ml.)	10 ml.
Distilled water	30 ml.
Adenosine triphosphate (disodium salt) *	152 mg.

As soon as the ATP is dissolved, adjust the pH to 9·4 with 0·1 M-NaOH and make up to 100 ml. with distilled water. Filter if turbid.

Method

(1) Incubate sections for 5 minutes to 3 hours at 37°.
(2) Wash in three changes of 1 per cent. $CaCl_2$.
(3) Transfer to 2 per cent. $CoCl_2$, 3 minutes.
(4) Wash in distilled water.
(5) Develop in dilute yellow ammonium sulphide.
(6) Wash, counterstain nuclei if desired (carmalum) or background (eosin). Dehydrate, clear, and mount in a suitable synthetic medium.

Result (Figs. 119 and 120)

Blank deposits indicate ATPase. Localization is good.

LEAD METHOD FOR ADENOSINE TRIPHOSPHATASE
(after Wachstein and Meisel)

(Unfixed frozen sections, free-floating or mounted; formalin-fixed frozen sections)

Preparation of the Incubating Medium

		Final concentration
20 ml.	125 mg. per cent. ATP (disodium salt)	$8{\cdot}3 \times 10^{-4}$ M
20 ml.	0·2 M-" tris "-HCl buffer (pH 7·2)	8×10^{-2} M
3 ml.	2 per cent. $Pb(NO_3)_2$	$3{\cdot}6 \times 10^{-3}$ M
5 ml.	0·1 M-$MgSO_4$	1×10^{-2} M
2 ml.	Distilled water.	

Add constituents in the above order and adjust the pH if necessary.

Method

(1) Incubate fresh or formalin-fixed sections for 5–60 minutes.
(2) Rinse in distilled water.
(3) Develop in 1 per cent. yellow ammonium sulphide, 1 minute.
(4) Rinse again in water.
(5) Mount in glycerine jelly.

Result (Fig. 122)

Brownish-black deposits indicate ATPase activity.

* Final concentration, 0·005 M.

METHOD FOR THIAMINE PYROPHOSPHATASE
(after Eränkö and Hasan)

(Cold microtome or cold-knife sections; free-floating or mounted)

Preparation of the Medium

Dissolve 60 mg. $Pb(NO_3)_2$ in 45 ml. 0·005 M-acetate buffer (pH5·0). Add 5 ml. 5 per cent. thiamine pyrophosphate. Incubate at 37° for 24 hours, filter and store in the cold, if necessary. Filter again before use.

Method

(1) Incubate slides at 42° for 5–60 minutes.
(2) Rinse in 1 per cent. acetic acid.
(3) Blot and dry at 60° for 5 minutes.
(4) Cover sections with a thin film of celloidin.
(5) Treat with dilute yellow ammonium sulphide, 2 minutes.
(6) Rinse in water.
(7) Mount in glycerine jelly.

Result

A brownish-black deposit indicates sites of thiamine pyrophosphatase activity.

METHOD FOR INORGANIC PYROPHOSPHATASE
(after Kurata and Maeda)

(Cold microtome or cold-knife sections, free-floating or mounted; cold formalin-fixed frozen sections)

Preparation of Substrate Solutions

Alkaline Pyrophosphatase. Dissolve 1·088 g. sodium pyrophosphate ($Na_4P_2O_7.12H_2O$) in 20 ml. distilled water. Prepare a 6 per cent. solution of ferric chloride ($FeCl_3.6H_2O$) and add 10 ml. to the above solution. A white precipitate forms. Add 10 per cent. sodium carbonate drop by drop until the precipitate just dissolves. Adjust the pH of the medium to 7·2 to 7·3 with N-HCl. Dilute to 100 ml. with distilled water and make hypertonic by adding 0·9 g. NaCl. This solution must be freshly prepared and, just before using, about 1 ml. of 10 per cent. magnesium chloride is added, drop by drop. Slight clouding of the medium can be ignored.

Acid Pyrophosphatase. Prepare the pyrophosphate-ferric chloride solution, as above, and centrifuge. Add the precipitate to a small amount (10 ml.) of distilled water and dissolve by adding 10 per cent. Na_2CO_3. Adjust the pH to 4·7 to 6·0 by adding N-HCl. Dilute to 100 ml. Allow to stand at 22° for 2–3 hours and readjust pH to 3·7 to 4·0 by adding 100 ml. 0·5 M-acetate buffer (pH 3·5).

Method

(1) Incubate 20 μ sections at 37° for 3 hours (alkaline medium) or for 1 hour (acid medium).

(2) Wash twice (10–15 minutes) in 0·9 per cent. NaCl.

(3) If free-floating sections have been used they should be mounted at this point.

(4) Treat with dilute yellow ammonium sulphide, 2 minutes (sections turn deep or light green).

(5) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic medium or in Canada balsam.

Result

A green deposit indicates sites of pyrophosphatase activity. The colour is not stable.

METHOD FOR INORGANIC POLYMETAPHOSPHATASE (after Berg)

(Freeze-substitution, Wolman; paraffin sections)

Preparation of Tissues

Quench small pieces of tissue in cold *iso*-pentane at — 160°. Transfer to acetone at — 78°, without thawing. Allow 14 days for dehydration. Subsequently the dehydrated specimens are brought to acetone at room temperature, cleared in benzene (30 minutes) and vacuum embedded in 56° paraffin wax. Tissues fixed in Wolman's 5 per cent. acetic-ethanol at — 4° for 1 hour can be used as an alternative.

Preparation of Substrate Solution

Stock Solution I. 0·07 M-lead acetate, 0·18 M-acetic acid and 0·045 M-nitric acid.

Stock Solution II. Freshly prepared 5·1 per cent. sodium metaphosphate.

Stock Solution III. 0·3 N-nitric acid.

Add 10 ml. Stock I and 1 ml. Stock II to 75 ml. water. Slowly add 4 ml. Stock II with constant stirring. Throughout the procedure the pH must be maintained at below pH 3.9 by addition of small amounts of Stock III. Finally filter with suction through a Whatman No. 50 paper.

Method

(1) Cut sections at 6μ. Pass through xylene and dilutions of dioxane to water (exposure to alcohol must be avoided).

(2) Incubate mounted or unmounted sections for 18–24 hours at 37°.*

(3) Rinse in 0·05 M-acetate buffer at pH 3·9.

(4) Develop with dilute yellow ammonium sulphide.

(5) Wash in water.

(6) Dehydrate in alcohol, clear with tetrachloroethylene and mount in a medium made up with this solvent in place of xylene.

Control sections inactivated by exposure to 90° for 10 minutes should be used.

Result

Brownish-black deposits indicate sites of polymetaphosphatase activity.

METHOD FOR GLUCOSE-6-PHOSPHATASE (after Chiquoine)

(Cold microtome or cold-knife sections; free-floating or mounted)

Conversion of Barium Salt of Glucose-6-phosphate to Potassium Salt

Dissolve 250 mg. of the barium salt in 10 ml. distilled water containing 2 drops of 2 N-HCl. Add 120 mg. potassium sulphate. Allow to stand for

* The original author recommends that a filtration accelerator pad (Fisher) should be placed in the incubating solution.

2 hours with frequent stirring. Centrifuge and test the supernatant for barium with a pinch of potassium sulphate. If no precipitation occurs dilute to 30 ml. with distilled water and adjust pH to 6·7 with N-KOH.

Preparation of the Incubating Medium

Dilute 1 part of the above solution with 2 parts of 0·006 M-lead nitrate (0·2 per cent. Filter before use.)

Method

(1) Incubate sections for 5–15 minutes at 32°.
(2) Wash in distilled water.
(3) Develop with dilute yellow ammonium sulphide.
(4) Wash in water, dehydrate, clear in xylene and mount in Clarite. (Alternatively mount in glycerogel without dehydration.)

Result

Brownish-black deposits indicate sites of glucose-6-phosphatase activity.

METHOD FOR GLUCOSE-6-PHOSPHATASE
(after Wachstein and Meisel)

(Cold microtome, free-floating sections)

Method

(1) Incubate 10–15 μ sections for 5–15 minutes at 32° in a substrate mixture consisting of 20 ml. of a 125 mg. per cent. solution of potassium glucose-6-phosphate, 20 ml. of 0·2 M-" tris " buffer (pH 6·7), 3 ml. of 2 per cent. lead nitrate, and 7 ml. distilled water.
(2) Wash in distilled water.
(3) Develop in dilute yellow ammonium sulphide.
(4) Wash in water.
(5) Post-fix in 6 per cent. neutral formaldehyde.
(6) Mount in glycerine and ring the coverslip with nail polish.

Result (Fig. 126)

Brownish-black deposits indicate sites of glucose-6-phosphatase activity.

APPENDIX 15

THE LEAD NITRATE METHOD FOR ACID PHOSPHATASE (after Gomori, 1950)

(Cold acetone, paraffin sections; cold formalin, frozen sections; cold microtome—post-fixed Wolman—sections)

Method

(1) Cut frozen sections 10–15 μ thick and mount them on slides. Alternatively, mount 4–8 μ cold microtome sections on coverslips and post-fix for 1–2 hours in cold acetone or in Wolman's fixative at 0–4°.

(2) If formalin-fixed frozen sections have been used they should be dried thoroughly to ensure adherence.

(3) Incubate at 37° for $\frac{1}{2}$–16 hours (average for most tissues 4 hours) in 0·01 M-sodium β-glycerophosphate in 0·05 M-acetate buffer (pH 5·0), containing 0·004 M-lead nitrate.

(4) Wash briefly and immerse in dilute yellow ammonium sulphide, 1–2 minutes.

(5) Wash and counterstain with 1 per cent. aqueous eosin, 5 minutes.

(6) Wash well, mount in glycerine jelly.

Result (Figs. 129, 132 and 134)

The presence of acid phosphatase in the sections is indicated by a black precipitate of lead sulphide.

MODIFIED LEAD NITRATE METHOD FOR ACID PHOSPHATASE (after Takeuchi and Tanoue)

(Cold acetone, paraffin sections; cold microtome, mounted sections; post-fixed)

Method

(1) Incubate sections for $\frac{1}{4}$–2 hours in the following :

2 vols. 2 per cent. sodium-β-glycerophosphate.
1 vol. 0·1 M-acetate buffer (pH 5·0–6·0).
1 vol. 2 per cent. lead acetate.
0·3 vol. 1–5 per cent. $MgCl_2$.

(2) Rinse in distilled water.

(3) Develop in ammoniacal silver nitrate solution for 30 minutes. (Add 28 per cent. ammonia water drop by drop to 5 per cent. aqueous $AgNO_3$ until the precipitate just dissolves).

(4) Rinse in 5 per cent. sodium thiosulphate for 5 minutes.

(5) Dehydrate, clear and mount in a suitable synthetic medium or mount directly in glycerine jelly.

Result

A brownish precipitate indicates sites of acid phosphatase activity.

STANDARD COUPLING AZO DYE TECHNIQUE FOR ACID PHOSPHATASE

(Cold microtome, unfixed or post-fixed; cold formalin, frozen sections)

Method

(1) Fix thin (2–4 mm.) slices of tissue in 10 per cent. neutral formalin at 4° for 10–16 hours.

(2) Cut frozen sections 10–15 μ thick and mount them on slides, without adhesive, drying for 2–3 hours at room temperature to ensure adherence. Alternatively use cold microtome sections, mounted on coverslips, fresh, or post-fixed in cold acetone or cold Wolman's fixative.

(3) Incubate the sections at 37° in the following mixture: Dissolve 10–20 mg. of sodium α-naphthyl phosphate in 20 ml. 0·1 M-veronal acetate buffer (Michaelis, 1931) at pH 5·0. (Alternatively 0·1 M-acetate buffer (Walpole) may be used.) Add 1·5 g. polyvinyl pyrrolidone and allow to dissolve. Add approximately 20 mg. of the stable diazotate of *o*-amino azotoluene (Fast Garnet GBC salt, I.C.I. Ltd.) or of diethylsulphamino-*o*-anisidine (Fast red ITR). Shake well and filter the mixture on to the dry sections. The incubation times vary : ½–1 minute for dog prostate, 30–60 minutes for rat liver.

(4) Wash in running water for 2 minutes.

(5) Counterstain in Mayer's hæmalum, 4–6 minutes.

(6) Wash in running water.

(7) Mount in glycerine jelly.

Result (Plates IVB and C ; Fig. 115)

Sites of acid phosphatase activity are coloured reddish-brown with salts 18 or 19 p. 871. With longer incubation periods the result is invariably crystalline. Nuclei, deep blue.

NAPHTHOL AS-BI PHOSPHATE METHOD (after Burstone)

(Freeze-dried or cold acetone fixed; paraffin sections)

Preparation of Incubating Medium

Dissolve 4 mg. Naphthol AS-BI phosphate * in 0·25 ml. dimethylformamide † and add 25 ml. 0·2 M-acetate buffer, pH 5·2–5·6. Add 35 mg. of a suitable diazonium salt (Red-violet LB salt, or salts 4 or 5) and 2 drops of 10 per cent. $MnCl_2$. Shake and filter.

Method

(1) Incubate sections, after removal of wax, for ½–6 hours at 37°.

(2) Wash in running water.

(3) Counterstain with Mayer's hæmalum, 1½ minutes.

(4) Wash in running water.

(5) Mount in glycerine jelly or in Burstone's PVP mounting medium (see p. 874).

Result (Fig. 136)

Sites of acid phosphatase activity appear in various shades of red. Nuclei blue.

* Alternative substrates are AS-TR and AS-MS phosphates.

† If frozen sections are being used, reduce this to 0·1 ml.

POST-COUPLING METHOD FOR ACID PHOSPHATASE
(after Rutenburg and Seligman)

(*Cold formalin; free-floating frozen sections; cold microtome sections, mounted, unfixed*)

Preparation of Sodium 6-benzoyl-2-naphthyl phosphate

Dissolve 14 g. 6-benzoyl-2-naphthol and 8·6 g. phosphorus oxychloride in 100 ml. dry benzene. Heat under reflux for a few minutes. Add 5 ml. dry pyridine and continue refluxing for 30 minutes. Cool and remove precipitate of pyridine hydrochloride by filtration. Remove the solvent by distillation on a water pump until a thick syrup is obtained. Do not allow the temperature to exceed 70–80°. Pour the warm syrup into a Petri dish in a dessicator over saturated aqueous KOH. Close the dessicator and apply low vacuum. In the course of several days crystals of the acid phosphate appear. Precipitate the monosodium salt by dissolving the acid phosphate in methanol and adding an equivalent of sodium methoxide in methanol. Yield 11·2 g. (60 per cent.). To remove sodium chloride and sodium phosphate suspend the solid in a few ml. of cold water, filter, wash with methanol and ether, collect and dry.

Preparation of Substrate Solution

Dissolve 25 mg. in 80 ml. distilled water and add 20 ml. 0·5 M-acetate buffer (pH 5·0). Make this solution hypertonic by adding 2 per cent. of solid NaCl.

Method

(1) Incubate sections, after brief washing in 0·8 per cent., 1·0 per cent. and 2·0 per cent. NaCl, for ½–2 hours (fixed sections) or for 10–60 minutes (fresh sections).

(2) Wash fresh sections in three changes of cold saline. Fixed sections can be washed in water.

(3) Transfer to a cold aqueous solution of diazonium salt * (1 mg./ml.), made alkaline with sodium bicarbonate, for 3–5 minutes.

(4) Wash in three changes of cold saline.

(5) Fix (unfixed sections) in cold formalin (10 per cent., 2 hours).

(6) Mount in glycerine jelly.

Result (Fig. 137)

A fine precipitate, blue or reddish-blue (Salt 6) or red (Salt 18), indicates sites of acid phosphatase activity.

THE LEAD NITRATE METHOD FOR PHOSPHOAMIDASE
(after Gomori, 1948)

(*Cold acetone; double-embedded sections*)

Preparation of Solutions

(1) **Stock Substrate.** Dissolve sufficient *p*-chloroanilidophosphonic acid to make a 0·1 M solution in an excess of 10 per cent. NH_4OH. Adjust the pH to about 8 by adding dilute acetic acid and make up the volume with distilled water. This solution is stable at 4° for at least a month.

* The authors recommend Fast blue B salt (6), but if a single-coloured end product is desired, salt 18 may be used instead.

(2) **Maleate Buffer.** Dissolve 5·8 g. of maleic acid in 500 ml. distilled water. Add 62 ml. N-NaOH and make up to 1,000 ml. The pH should be in the neighbourhood of 5·6.

(3) **0·1 M-Lead Nitrate.**

(4) **Ten per cent. Manganese Chloride.**

(5) **The Incubating Medium.** Add 2 ml. of 0·1 M stock substrate solution to 50 ml. maleate buffer with 1·5 ml. of 0·1 M-lead nitrate and a few drops of $MnCl_2$. Incubate at 60° for 30 minutes and filter into a Coplin jar.

Method

(1) Incubate mounted frozen sections for 2–4 hours, keeping the Coplin jar at an angle and the slides face downwards.

(2) Rinse in distilled water, wipe precipitate from around sections and from the backs of the slides.

(3) Rinse in 0·1 M-citrate or acetate buffer at pH 4·5 until the diffuse white precipitate covering the section disappears.

(4) Rinse in running water.

(5) Treat with dilute yellow ammonium sulphide solution, 1–2 minutes.

(6) Wash in running water.

(7) Counterstain in 1 per cent. aqueous eosin, 3–5 minutes.

(8) Wash well and mount in glycerine jelly.

Result (Fig. 138)

A black precipitate is presumed to indicate phosphoamidase activity.

MODIFIED PHOSPHOAMIDASE TECHNIQUE
(after Meyer and Weinmann)

(Cold acetone; double-embedded sections)

Preparation of Incubating Medium

(1) **Stock Solution A.** Dissolve 2·08 g. *p*-chloroanilidophosphonic acid in 15 ml. N-NaOH and make up to 100 ml. with distilled water.

(2) **Solution B.** Dissolve 534 mg. maleic acid in 5 ml. N-NaOH and make up volume to 100 ml. with distilled water. Add 175 mg. NaCl and 94 mg. $Pb(NO_3)_2$. The mixture becomes turbid and should be heated gently until it clears. Add 4·5 ml. of Stock Solution A and heat to 44°. Filter. The filtrate should be clear.

Method

(1) Mount two (serial) sections, one at either end of the slide.

(2) Immerse one of the sections in 10 per cent. nitric acid for 90 minutes, without removal of paraffin wax.

(3) Wash this section and blot dry.

(4) Remove wax from both sections with xylene.

(5) Incubate slides for $1\frac{1}{2}$–4 hours at 42°, in a horizontal position with the sections facing downwards. The authors recommend a rectangular bath and 7 mm. solution below and 4 mm. above.

(6) Differentiation. Sections incubated for over 3 hours require treatment with 0·1 M-citric acid until the inactivated section is clear of precipitate.

(7) Wash in water and treat with dilute yellow ammonium sulphide for 2 minutes.

(8) Wash in water, dehydrate, clear and mount in a suitable synthetic medium.

Result

A brownish-black precipitate indicates sites of phosphoamidase activity.

PREPARATION OF NAPHTHOL AS-TR AND AS-BI PHOSPHODIAMIDES
(after Burstone)

Prepare the dichlorides of Naphthol AS-TR or Naphthol AS-BI in the manner detailed in Appendix 14, p. 873. Treat a small quantity (1 g.) of the chosen naphthol dichloride in tetrahydrofurane with excess NH_4OH. A gelatinous precipitate forms which is discarded. The supernatant is evaporated to dryness. Fine crystals of the diamide are formed.

Details of yield, etc., have not been given.

APPENDIX 16

THE α-NAPHTHYL ACETATE METHOD FOR ESTERASE

(cold formalin, frozen sections; cold acetone, paraffin sections; cold microtome, formalin post-fixed sections)

Method

(1) Cut frozen sections 10–15 μ thick and mount on clean slides. Dry in air to ensure adherence.

(2) Incubate for 1 to 15 minutes at room temperature in the following medium : Dissolve 10 mg. α-naphthyl acetate in 0·25 ml. acetone and add 20 ml. 0·1 M-phosphate buffer (pH 7·4).* Shake thoroughly until most of the initial cloudiness disappears. Add 50–100 mg. Fast B salt (I.C.I. Ltd.),† shake, and filter directly on to the sections. These should be dry if frozen sections but brought to water and left wet if paraffin sections.

(3) Wash in running water, 2 minutes.

(4) Counterstain in Mayer's hæmalum, 4–6 minutes.

(5) Wash in running water for at least 30 minutes.

(6) Mount in glycerine jelly.

Result (Figs. 141 and 142 ; Plate IVD)

Esterase, black ; nuclei, dark blue. (Lipase, AChE and ChE can also hydrolyse α-naphthyl acetate and appear black, see Chapter XVI, pp. 482–486, for methods of distinguishing.)

THE NAPHTHOL AS ACETATE METHOD FOR ESTERASE (after Gomori)

(cold formalin, frozen sections; cold acetone, paraffin sections; cold microtome, formalin post-fixed sections)

Preparation of the Substrate

Dissolve 5 g. 2-hydroxy-3-naphthoic anilide (Naphthol AS, or Brenthol AS, I.C.I. Ltd.) in 10 ml. dry pyridine with 20 ml. acetic anhydride in addition. Heat under a reflux condenser for 1 hour. Pour into cold water, filter off the pasty product and dry. Recrystallize from ethanol (containing a little charcoal) to obtain the acetate as a cream-coloured powder (m.p. 160°–161°).

Method

(1) Cut frozen sections at 10 μ and mount on clean slides, dry in air to ensure adherence.

(2) Add 0·1 ml. 1 per cent. Naphthol AS acetate to 10 ml. 0·05 M-phosphate buffer at pH 7·0. Shake to form a slightly turbid medium and add

* Alternatively, add 1·0 ml. 1 per cent. α-naphthyl acetate in acetone to 10 ml. of the buffer.

† Other diazonium salts can be used for coupling. I have tried the twenty-two salts which appear in the Table on page 870 ; the majority of these give an orange or red particulate deposit. In no case was the result superior to that obtained by the method given above but, if it seems necessary to check the occurrence of diffusion into lipid tissue components, control sections should be incubated with a diazonium salt giving a particulate result.

10 mg. of a suitable stable diazotate.* Stir, and filter on to the dry section.

(3) Incubate at 17°–22° for 20–30 minutes.

(4) Wash in running water for 1 minute.

(5) Counterstain, according to need, with either Mayer's hæmalum, 4–6 minutes, or with carmalum, 1–6 hours.

(6) Mount in glycerine jelly.

Result (Fig. 144, Plate Va)

A particulate azo dye is deposited at sites of esterase activity. Nuclei, blue or red.

Gomori (1952) recommended the diazotate of *o*-aminoazotoluene ; this gives a finely particulate orange-red precipitate. He also recommended the use of cold acetone-fixed paraffin sections and the technique given above can equally well be employed for these. The necessary incubation time is longer (1–1$\frac{1}{3}$ hours at room temperature) but, because diffusion is less likely than with the α-naphthyl acetate method, the Naphthol AS method is well suited to use with acetone-fixed paraffin sections. Naphthol AS is particularly sensitive to pH changes when coupling with diazonium salts. The best results are obtained within the range of pH 6·8–7·2.

NAPHTHOL AS-LC ACETATE METHOD FOR ESTERASE (after Burstone)

(cold acetone or freeze-dried, paraffin sections; cold formalin, frozen sections)

Preparation of the Substrate

Suspend 5 g. naphthol AS-LC in 50 ml. tetrahydrofuran and add 4 ml. acetic anhydride, and then 4 ml. of 5 N-NaOH with constant stirring until the solution becomes clear. Pour the clear supernatant into light petroleum and collect the precipitate under suction. Dry, dissolve in hot methanol, filter, and recrystallize several times from cold methanol. (Naphthol AS-LC acetate ; m.w. 399·82 ; m.p. 138°).

Method (paraffin sections) †

Dissolve 20 mg. of the substrate in 4 ml. dimethylformamide and add 5–10 ml. ethyl cellosolve (ethylene glycol monoethyl ether) and 10 ml. 0·2 M-tris buffer (pH 7·1). Make up to 50 ml. with distilled water. Add 30 mg. of diazonium salt (Garnet GBC, or Corinth LB), shake, filter and use at room temperature. The addition of a few drops of 20 per cent. Tween 20 is sometimes helpful.

(2) Incubate sections for 10–40 minutes.

(3) Wash in running water.

* Of the twenty-two stable diazotates listed in the Table on page 870, only four give a satisfactory result. These are the diazotates of 4-benzoylamino-2 : 5-dimethoxyaniline, 4-chloro-*o*-anisidine, 5-chloro-*o*-toluidine, and *o*-aminoazotoluene. The first gives a steel blue precipitate of moderate size (Fig. 144) which does not obscure cellular detail, the second gives a very fine particulate dull red precipitate (Plate Va), and the third a bright scarlet precipitate in which the particle size is too large for convenience even with low magnification. The fourth gives a fine orange-red precipitate.

† A somewhat different medium should be used for frozen sections. Burstone recommends placing 3–5 mg. of substrate in a 50-ml. flask. After adding 2 ml. dimethylformamide and 5 ml. ethyl cellosolve the volume is made up by adding distilled water and buffer (pH 7·2 to 7·4). This solution should not be filtered but 40 mg. Garnet GBC salt in 3 ml. water should be filtered into it. Sections are preferably used free-floating.

(4) Counterstain nuclei with Mayer's hæmalum, 1½ minutes.

(5) Wash in running water, mount in glycerine jelly or in 80 per cent. alcoholic polyvinyl acetate.

Result

Esterases produce a bright red precipitate ; nuclei blue.

THE INDOXYL ACETATE METHOD FOR ESTERASES (Holt and Withers, 1952; Holt, 1958)

(cold formalin, frozen sections; cold acetone, cold formalin and acetone, paraffin sections)

Preparation of the Incubating Medium

O-acetyl-5-bromoindoxyl* . .	1·3 mg.	
Ethanol	0·1 ml.	
Allow to dissolve and add :		*Final concn.*
0·1 M tris(hydroxymethyl)amino-methane/HCl buffer (pH 6–8) .	2·0 ml.	0·02 M
0·05 M-potassium ferricyanide .	1·0 ml.	5×10^{3} M
0·05 M-potassium ferrocyanide .	1·0 ml.	5×10^{-3} M
0·1 M-calcium chloride. . .	1·0 ml.	0·01 M
Add water to 10 ml.†		

This medium should be freshly prepared.

Method

(1) Fix thin pieces of tissue in 5 per cent. neutral formaldehyde in 1 per cent. saline, at 4° for 24 hours. Alternatively use 4 per cent. formaldehyde containing 1 per cent. $CaCl_2$.

(2) Avoid washing in water. This causes loss of enzyme.

(3) Cut frozen sections at 10–15 μ and transfer these to the incubating medium, at 22° or 37°, for 10 seconds to 15 hours. (Development can be carried out under inspection, using the microscope for small structures. Within fairly broad limits over-incubation is not dangerous since it only increases the intensity without altering the localization.)

(4) Counterstain the nuclei, if desired, in Mayer's Carmalum, 10 minutes.

(5) Rinse briefly in water.

(6) Mount sections on clean dry slides.

(7) Either mount directly in glycerine jelly or dehydrate gently in 50 per cent., 70 per cent. and 95 per cent. alcohol, clear in carbol-xylene (1 part phenol, 3 parts xylene) and mount in Canada balsam.

Result (Figs. 146, 147 and Plates VB and C)

A blue precipitate of indigo forms at the site of esterase activity. Nuclei (if stained), deep red.

THE TWEEN METHOD FOR LIPASE (after Gomori)

(cold formalin, frozen sections; cold acetone, paraffin sections)

A large number of substrates have been employed for this method. Gomori recommended as best for most purposes either Tween 60 (Atlas

* Use alternatively, *O*-acetyl-4-chloro-5-bromoindoxyl, 1·5 mg., or *O*-acetyl-5-bromo-6-chlorindoxyl, 3·0 mg.

† Holt uses 10 ml. 1·0 M-NaCl in place of distilled water.

Powder Co.), the stearic ester of a polymannitol, or Product 81 (Onyx Oil and Chemical Co.), the stearic ester of a polyglycol. To demonstrate his "true lipase" Gomori recommended Tween 80, the oleic acid ester of a polymannitol, or G-6486, a ricinoleic acid ester.

Stock Solutions

(1) Five per cent. Tween 60 (Tween 80 for "true lipase").

(2) 0·5 M-tris(hydroxymethyl)aminomethane buffer pH 7·2–7·4 (Appendix 1). This substance was at one time not easily available in this country. In its place others employed 0·2 M-veronal acetate buffer (Mark, 1950) or bicarbonate buffer (Sneath, 1951).

(3) Ten per cent. $CaCl_2$.

Substrate and buffer solutions are preserved with 0·25 per cent. chloretone or with a crystal of thymol.

Incubating Medium

5 ml. buffer.
2 ml. $CaCl_2$.
2 ml. Tween solution.
40 ml. distilled water.

With a crystal of thymol included, this working solution keeps well in the refrigerator.

Method

(1) Cut frozen sections and mount on slides.

(2) After drying to ensure adherence, incubate for 3–12 hours in the above medium.

(3) Wash thoroughly in distilled water.

(4) Immerse in 1 per cent. lead nitrate for 15 minutes.

(5) Wash in running water, 5 minutes.

(6) Immerse in dilute yellow ammonium sulphide, 1–2 minutes.

(7) Wash and counterstain with 1 per cent. aqueous eosin, 5 minutes.

(8) Wash well, mount in glycerine jelly.

Result (Fig. 149)

A brownish-black deposit indicates the presence of lipase. (In my hands satisfactory results were obtained only with sections of pancreas.)

MODIFIED TWEEN METHOD FOR LIPASE-ESTERASE (after Martin)

(*formalin-fixed frozen sections; cold acetone, paraffin sections*)

Preparation of Stock Solution

Dissolve 2 per cent. Tween 40 in 0·2 per cent. $CaCl_2$ in veronal-acetate buffer (pH 7·4). Add a crystal of thymol. Incubate at 37° for 2 days and filter through a Seitz filter.

Preparation of Incubating Medium

0·05 M-veronal acetate (pH 7·2 to 7·6)	30 ml.
2 per cent. $CaCl_2$	2–3 ml.
Glycerol	10–20 ml.
Stock solution	2–4 ml.
Thymol	A small crystal

Method

Proceed as in Gomori method (above).

THE MYRISTOYL CHOLINE METHOD FOR CHOLINESTERASES (after Gomori)

(*cold formalin, frozen sections; cold acetone, paraffin sections*)

Preparation of Solutions

Stock Solution 1. 0·1 M-veronal-acetate buffer pH 7·6, 50–100 ml.
0·1 M-cobaltous acetate, 30–50 ml.
Distilled water to make up 300 ml.
Add 1 mg. each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

Add a crystal of thymol and store at 4°.

Stock Solution 2. 0·02 M-myristoyl choline in distilled water.

Add a crystal of thymol and store at 4°.

For use, add 1 ml. of stock solution 2 to 50 ml. of stock solution 1, previously heated to 37°.

Method

(1) Cut frozen sections 10–15 μ thick and mount on clean slides (alternatively, bring paraffin sections to water).

(2) Incubate for 1–4 hours (frozen sections), 2–16 hours (paraffin sections), in the working solution of myristoyl choline.

(3) Wash in running water, 2 minutes.

(4) Immerse in a dilute solution of yellow ammonium sulphide, 1–2 minutes.

(5) Wash in water.

(6) Counterstain in 1 per cent. aqueous eosin or 1 per cent. light green.

(7) Wash in water.

(8) Mount in glycerine jelly (frozen sections) or dehydrate rapidly, clear, and mount in Canada balsam (paraffin sections).

Result (Figs. 150 and 151)

Sites of ChE, and possibly some non-specific esterases, appear black.

THE ACETYLTHIOCHOLINE METHOD FOR CHOLINESTERASES (after Gomori (1952)

(*cold formalin, frozen sections; cold microtome, free-floating sections*)

Preparation of Stock Solutions

Copper sulphate ($CuSO_4.5H_2O$)	0·3 g.
Glycine	0·375 g.
Magnesium chloride ($MgCl_2.6H_2O$)	1·0 g.
Maleic acid	1·75 g.
NaOH (4 per cent.)	30 ml.
Na_2SO_4 (40 per cent.; hot saturated)	170 ml.

This solution keeps indefinitely (pH 6·0).

Preparation of Incubating Medium

Dissolve about 20 mg. acetylthiocholine iodide in a few drops of water. Add 10 ml. stock solution.

Method

(1) Incubate sections for 10–60 minutes at 37°.
(2) Rinse in 3 changes of saturated Na_2SO_4.
(3) Treat with dilute yellow ammonium sulphide, 2 minutes.*
(4) Wash briefly and mount in glycerine jelly.

Result (Fig. 152)

Sites of cholinesterase activity appear in shades of brown.

This method is much simpler than the Koelle method. Some workers may still prefer the latter, however, and directions therefore appear below.

THE ACETYL- AND BUTYRYLTHIOCHOLINE METHOD FOR CHOLINESTERASES (after Koelle)

(*fresh frozen*)

Preparation of Solutions

Storage Solutions

(1) **DFP**	Distilled water	4·5 ml.
	40 per cent. Na_2SO_4 . . .	9·0 ml.
	10^{-6} M-DFP	1·5 ml.

The 10^{-6} M-DFP solution was prepared within 30 minutes of the time of use by serial dilution of a 0·1 M stock solution in propylene glycol.

(2) **Storage Solution C**

Distilled water	4·5 ml.
40 per cent. Na_2SO_4 . . .	10·5 ml.

(3) **Storage Solution B-D**

Distilled water	6·0 ml.
40 per cent. Na_2SO_4 . . .	9·0 ml.

Storage solutions were kept at 35° and used at this temperature.

Incubating Solutions

The following Table gives the composition of the final incubation media for localization of AChE and ChE.

Solution	Enzyme localized	Cu-Gl ml.	H_2O ml.	Mal ml.	Na_2So_4 ml.	$MgCl_2$ ml.	CuThCh	AThCh ml.	BuThCh ml.
B	AChE	0·6	2·1	1·5	9·0	0·6	trace	1·2	—
C	ChE	0·6	0·6	1·5	10·5	0·6	trace	—	1·2
D	Control	0·4	1·4	1·0	6·0	0·4	trace	—	0·8

All solutions allowed to incubate 15 minutes after addition of CuThCh ; substrates (AThCh and BuTuCh) added immediately prior to filtering and introducing slides.

The solutions from which the final incubating media are made up are composed as follows :

(1) Copper-glycine (Cu-Gl) : 3·75 g. glycine, 2·5 g. $CuSO_4$, $5H_2O$, distilled water 100 ml.

* In Holmstedt's modification this step is omitted.

(2) Maleate buffer (Mal.) : 9·6 g. sodium hydrogen maleate, 52·2 ml. N-NaOH, distilled water to 100 ml.

(3) Sodium sulphate (Na_2SO_4) : 40 per cent. (W/v) Na_2SO_4 adjusted to pH 6·0, stored at 37°.

(4) Magnesium chloride ($MgCl_2$) : 9·52 g. $MgCl_2$ in 100 ml. distilled water.

(5) Acetylthiocholine (AThCh) : 23 mg. acetylthiocholine iodide, 1·2 ml. distilled water, 0·4 ml. 0·1 M-$CuSO_4$, $5H_2O$; centrifuged and the supernatant decanted for use.

(6) Butyrylthiocholine (BuThCh) : 43 mg. butyrylthiocholine iodide, 1·8 ml. distilled water, 0·6 ml. 0·1 M-$CuSO_4$, $5H_2O$; centrifuged, etc., as above.

(7) Copper thiocholine (CuThCh) : This is initially prepared by alkalinizing a solution of AThCh in copper-glycine solution to pH 12·0 with KOH, allowing to stand overnight at room temperature, collecting the precipitate and washing with water. Subsequently, the incubating solutions, after use, are filtered, allowed to stand at 37° for 2–4 days to permit spontaneous hydrolysis of the substrate, and the precipitate is collected and washed with water.

Method

(1) Following a minimal period of storage in the appropriate storage solutions, transfer the mounted sections into incubating solutions for 5–60 minutes at 37° (average time, 30 minutes).

(2) Transfer to rinsing solution I (20 per cent. Na_2SO_4, saturated with CuThCh) for 5 minutes.

(3) Transfer to rinsing solution II (10 per cent. Na_2SO_4, saturated with CuThCh) for 1 minute.

(4) Transfer to rinsing solution III (water, saturated with CuThCh) for 1 minute.

(5) Treat with CuS-saturated ammonium sulphide solution (dilute yellow ammonium sulphide treated with a few drops of $CuSO_4$, shaken and filtered) for 20 seconds.

(6) Rinse rapidly in water.

(7) Fix for 30 minutes in 10 per cent. neutral formalin, saturated with CuS.

(8) Dehydrate in CuS-saturated alcohols and clear in CuS-saturated xylene.

(9) Mount in Canada balsam.

Result

A dark brown precipitate indicates sites of cholinesterase activity. In sections incubated with solution B this indicates AChE, in those incubated with solution C, ChE. Control sections treated with DFP and incubated with solution D should be blank.

MODIFIED THIOCHOLINE AND INHIBITOR METHOD (after Holmstedt)

(fresh frozen, cold microtome, mounted sections)

Stock Solutions

(1) N-NaOH

(2) 0·1 M-$CuSO_4$

(3) 40 per cent. Na_2SO_4 adjusted to pH 6·0.

(4) 0·04 mM-Mipafox.
(5) 0·5 mM-284C51.
(6) Copper glycinate (3·75 g. glycine and 2·5 g. $CuSO_4 . 5H_2O$ in 100 ml. water).
(7) Maleate buffer, pH 6·0 (9·6 g. sodium hydrogen maleate with 50 ml. N-NaOH in 100 ml. water).
(8) $MgCl_2$, 9·52 per cent. aqueous solution.
(9) Acetylthiocholine sulphate (45 mg. acetylthiocholine iodide with 0·8 ml. 0·1 M-$CuSO_4$ and 2·4 ml. water ; precipitate discarded).
(10) butyrylthiocholine sulphate (50 mg. butyrylthiocholine iodide treated as above).
Solutions 3, 6, 7 and 8 should be stored in an incubator at 38°.

Method

(1) Cut fresh cryostat sections 10–15 μ thick and mount on slides or coverslips.
(2) Place sections in 25 per cent. Na_2SO_4 until required for use (as short a time as possible).
(3) Incubate a series of 7 sections as shown in the Table below.

Stage 1		Storage			
	Slides . .	ABCD		EFG	
	Solutions . .	St_1		St_2	
Stage 2		Incubation with inhibitor (30 mins. 37°)			
	Slides . .	BD	F	C	G
	Solutions . .	I_1	I_2	I_3	I_4
Stage 3		Wash (10 mins.)			
	Slides . .	BD	F		
	Solutions . .	St_1	St_2		
Stage 4		Re-incubation with inhibitor (30 mins. 37°)			
	Slides . .	D			
	Solutions . .	I_3			
Stage 5		Incubation with substrates (5–120 mins. 37°)			
	Slides . .	AB	CD	EF	G
	Solutions . .	S_1	S_2	S_3	S_4

Solutions indicated in the above table are composed as follows :

Solution	Amounts (ml.) of Stock Solutions								Amount of Water (ml.)
	3	4	5	6	7	8	9*	10*	
St_1	9.0	—	—	—	—	—	—	—	6·0
St_2	10·5	—	—	—	—	—	—	—	4·5
I_1	9.0	1·5	—	—	—	—	—	—	4·5
I_2	10·5	1·5	—	—	—	—	—	—	3·0
I_3	9·0	—	0·15	—	—	—	—	—	5·85
I_4	10·5	—	0·15	—	—	—	—	—	4·35
S_1	9·0	—	—	0·6	1·5	0·6	1·2	—	2·1
S_2	9·0	—	0·15	0·6	1·5	0·6	1·2	—	1·95
S_3	10·5	—	—	0·6	1·5	0·6	—	1·2	0·6
S_4	10·5	—	0·15	0·6	1·5	0·6	—	1·2	0·45

*Add just before use.

(4) Examine slides without further treatment.

Result

The precipitate of copper thiocholine sulphate appears as a microcrystalline deposit visible by ordinary microscopy. Interpretation of results is given below :

Slide	Treatment		Cholinesterase Indicated
	Inhibitor	Substrate	
A	—	AcThCh	AChE + BuChE
B	Mipafox	AcThCh	AChE
C	284C51	AcThCh	BuChE
D	Both	AcThCh	Nil
E	—	BuThCh	BuChE
F	Mipafox	BuThCh	Nil
G	284C51	BuThCh	BuChE

My own preference, as stated in Chapter XVI, is for 62C47 and *iso*OMPA in place of Holmstedt's pair. There is no reason why the two should not be substituted in the above scheme.

THE THIOLACETIC ACID METHOD FOR CHOLINESTERASE (after Crevier and Bélanger)

(*fresh, cold microtome sections; formalin-fixed frozen sections*)

Preparation of Incubating Medium

Dissolve 0·12 M-thiolacetic acid (CH_3COSH) (0·75 g.) and 0·0001 M-lead nitrate (27 mg.) in 83 ml. Na_2HPO_4 (0·1 M). Add 17 ml. McIlvaine's Phosphate-Citrate buffer (pH 6·2).

Method

(1) Incubate for 30–60 minutes at 22°.
(2) Wash in ice water at 4°.
(3) Counterstain nuclei, if desired, with 0·02 per cent. basic fuchsin.
(4) Wash in cold water, dehydrate, clear in xylene and mount in Permount (Fisher) or other suitable synthetic medium. (I prefer to avoid dehydration and to mount in glycerine jelly.)

Result

A black deposit indicates sites of cholinesterase activity.

APPENDIX 17

FERRIC HYDROXYQUINOLINE METHOD FOR BETA-GLUCURONIDASE

(after Fishman & Baker)

(*Cold Chloral-formalin, frozen sections; cold microtome, post-fixed sections*)

Preparation of Substrate Solution

Stage 1. Dissolve 50 mg. 8-hydroxyquinoline in 30 ml. 0·1 N-acetate buffer (pH 5·0) by warming on a boiling water-bath. Prepare a solution of ferric sulphate ($Fe_2(SO_4)_3.6H_2O$) by dissolving 1·522 g. in 100 ml. distilled water, warming as necessary. Add 20 ml. of this solution to the solution of 8-hydroxyquinoline, shake and maintain at 37° for 2 hours. Centrifuge at 2,000 r.p.m. for 15 minutes.

Stage II. Place 100 mg. 8-hydroxyquinoline glucuronide in a 125 ml. Erlenmeyer flask and add 2 ml. 1·0 M-acetate buffer (pH 5·0). Heat to boiling on a water-bath. Decant the solution from Stage I into this solution, centrifuge for 15 minutes and filter through Whatman No. 2 paper into a glass-stoppered bottle. Add to each 100 ml. 1 g. powdered gum acacia by dropping the powder, without stirring, on to the surface of the medium. Store at 0–4° in a refrigerator.

Preparation of Other Reagents

Oxalate buffer. (0·5 M) Sodium oxalate, 2·87 g., oxalic acid, 0·47 g., distilled water, 100 ml.

Neutral Red. 0·5 per cent. aqueous solution.

Potassium ferrocyanide. 2·0 per cent. aqueous solution.

Method

The original authors recommend that thin (2–3 mm.) tissue blocks should be fixed in not less than 30 times their own volume of chloral-formalin at 8° for 17–24 hours. This fixative is prepared by adding 0·1 g. chloral hydrate to 20 ml. neutral formaldehyde (40 per cent.) and making up to 100 ml. with distilled water. For friable tissues a gelatine-embedding procedure is advised.

(1) Cut 10–20 μ sections on the freezing microtome.

(2) Place sections in substrate solution in 6 ml. capacity watch glasses, up to one average section per ml. Avoid overlapping and folding.

(3) Transfer to refrigerator at 8° for 17–24 hours.

(4) Incubate at 37° for $\frac{1}{2}$ to 7 hours.

(5) On removal of the watch glass from the incubator skim the surface of the medium with filter paper until all the surface deposit is removed.

(6) Transfer sections with a glass needle to distilled water. Mount on a clean glass slide and examine under the microscope. If the reaction is inadequate return the section to the incubating medium. If adequate, return section to distilled water.

(7) Place sections in oxalate buffer for 15 minutes at room temperature.

(8) Wash well in distilled water.

(9) Pick up section on albuminized slide and carefully blot dry.
(10) Dry at 37° for 5–10 minutes.
(11) Rinse in distilled water.
(12) Cover section with 6 drops of the ferrocyanide solution. Add 6 drops of N-HCl and mix by tilting. Leave for 2 minutes.
(13) Flood the slide with distilled water and repeat stage 12 for a further 10 minutes.
(14) Wash in several changes of distilled water.
(15) Counterstain nuclei with neutral red for $\frac{1}{2}$–2 minutes.
(16) Flood slide repeatedly with distilled water.
(17) Dehydrate in alcohol, clean in xylene, followed by toluene, and mount in Permount.

Result (Figs. 161 and 162 ; Plates VIb and c)

A microcrystalline deposit of Prussian blue indicates sites of β-glucuronidase activity. Nuclei red.

Biosynthesis of 8-hydroxyquinoline glucuronide (m.p. 154–156°)

Young rabbits maintained on a diet of oats and water should be housed in metabolism cages. Pure 8-hydroxyquinoline is mixed with peanut oil (1 g. per 2 ml.) in a glass tissue homogenizer. The product is administered by stomach tube. The urine is filtered through cotton gauze, adjusted to pH 4·0 with HCl, and placed in a refrigerator. After 24 hours the crop of greenish crystals is removed by filtration. The product is purified by solution in the minimal quantity of hot water, shaking with powdered charcoal and filtering.

It is essential that the rabbits secrete an acid urine since the glucuronide is unstable in the alkaline pH range.

8-HYDROXYQUINOLINE COUPLING AZO DYE METHOD

(*cold formalin, frozen sections*)

Method

(1) Cut frozen sections 10–15 μ and mount on clean glass slides. Dry in air to ensure adherence. Ring the sections with paraffin wax so as to enclose a small pool of the incubating medium.
(2) To 10 ml. 0·003 to 0·005 M-8-hydroxyquinoline glucuronide in 0·1 M -acetate buffer pH 5·2 add 10 mg. of the stable diazotate of 4-benzoylamino-2: 5-dimethoxyaniline (I.C.I. Ltd.). Shake the mixture and filter directly on to the dry slides.
(3) Incubate for 2–4 hours in a closed dish in which is placed some wet blotting paper to minimize evaporation.
(4) Wash in running water and remove the ring of wax at the same time.
(5) Counterstain in Mayer's hæmalum, 4–6 minutes.
(6) Wash in running water for 30 minutes.
(7) Mount in glycerine jelly.

Result (Fig. 159)

An orange precipitate is deposited in the neighbourhood of structures containing β-glucuronidase.

POST-COUPLING METHOD FOR BETA-GLUCURONIDASE

(after Seligman, Tsou, Rutenburg and Cohen)

(*Cold microtome, mounted sections, post-fixed in neutral formalin*)

Preparation of Substrate Solution

Dissolve 30 mg. 6-bromo-2-naphthyl-β-D-glucopyruronoside (glucuronide) in 5 ml. absolute ethanol and add 20 ml. phosphate-citrate buffer (pH 4·95) and 75 ml. water.

Method

(1) Incubate sections for 4–6 hours in the substrate solution at 37°.
(2) Rinse in tap water.
(3) Immerse in a solution of Fast blue B (6) in cold (4°) 0·02 M-phosphate buffer (pH 7·5). A strength of 1 mg. per ml. is adequate.
(4) After 2 minutes remove sections, wash in two changes of cold distilled water.
(5) Rinse in 0·1 per cent. acetic acid.
(6) Mount in glycerine jelly.

Result (Fig. 160)

A blue or purple colour indicates sites of high β-glucuronidase activity. A red colour may indicate lower activity or the presence of lipid.

POST-COUPLING METHOD FOR BETA-GALACTOSIDASE *

(after Rutenburg *et al.*, 1958)

(*Cold microtome, mounted sections; cold formalin, frozen sections*)

Preparation of Substrate Solution

Dissolve 100 mg. 6-bromo-2-naphthyl-β-D-galactopyranoside (galactoside) in 15 ml. methanol and add 200 ml. hot distilled water (70°). Allow the solution to cool and add 85 ml. phosphate-citrate buffer (pH 4·95). Prepare the buffer from equal volumes of 0·2 M-disodium phosphate and 0·1 M-citric acid. Add a further 100 ml. distilled water. The solution is stable for 6 months at 4°.

Method

(1) Incubate sections at 37° in the above solution. Active tissues need 1–4 hours. Tissues containing only weak enzyme activity may be incubated for 8 hours or more at 25°.
(2) Wash in 3 changes of distilled water, for 3 minutes in each.
(3) Transfer to a freshly prepared solution of Fast blue B (6), 1 mg./ml., at 4° and at pH 7·4 to 7·8. Agitate gently for 3–5 minutes.
(4) Wash 3 times in cold water.
(5) Mount in glycerine jelly.

Result (Fig. 164)

A blue or purple colour indicates sites of β-galactosidase activity.

* A similar method can be used for β-glycosidase using 6-bromo-2-naphthyl-β-D-glycoside as substrate.

POST-COUPLING* METHOD FOR ALPHA-GLUCOSIDASE

(after Rutenburg, Lang, Goldburg, Rutenburg)

(*Cold neutral formalin, frozen sections*)

Method

(1) Dissolve 1 to 1·5 mg. 6-bromo-2-naphthyl-α-D-glucopyranoside (glucoside) in 1 ml. ethanol and add 9 ml. 0·025 M-phosphate buffer (pH 6·5).

(2) Incubate free-floating sections for 1–2 hours at 37°.

(3) Rinse in water.

(4) Treat with coupling solution (1 mg./ml. Fast blue B in phosphate buffer at pH 7·5) for 2 minutes.

(5) Wash in water and mount in glycerine jelly.

Result

Blue or purple dyes indicate sites of enzyme activity.

METHOD FOR N-ACETYL-BETA-GLUCOSAMINIDASE

(after Pugh and Walker)

(*Cold formalin, frozen sections; cold microtome, post-fixed sections*)

Method

(1) Fix thin slices of tissue in neutral formalin or formol-calcium for 12 hours at 4°.

(2) Incubate for 20–60 minutes at room temperature in the following incubation medium.

Saturated aqueous α-naphthyl-acetyl-β-glucosamine (1mg/ml.)	1·0 ml.
Phosphate-citrate buffer (pH 4·6)†	0·1 ml.
Fast Garnet GBC salt (18) (15mg./ml., filtered)	0·1 ml.

(3) Wash in water.

(4) Counterstain nuclei, if required, with haemalum.

(5) Mount in glycerine jelly.

Result (Fig. 165)

A fine crystalline or diffuse reddish-brown azo dye indicates sites of enzyme activity.

METHOD FOR GLUCOSAN PHOSPHORYLASE AND TRANSGLYCOSYLASE

(after Takeuchi)

Preparation of Substrate Solutions

Stock Solution

Dissolve 50 mg. glucose-1-phosphate (potassium salt) 10 mg. adenosine-5-phosphate and 2 mg. glycogen in 15 ml. distilled water. Add 10 ml. 0·1M-acetate buffer (pH 5·6 to 6·0), and 1 drop of insulin (20 units/ml).

* The method may be used as a simultaneous coupling procedure by inclusion of suitable diazonium salts in the incubating medium.

† Citric-acid, 0.1M, 10.65 ml.
$Na_2HPO_4.12H_2O$, 0.2M, 9.35 ml.

This mixture keeps for less than a week. If the glycogen is omitted it can be stored for longer periods and a knife-point of glycogen can be added immediately before incubation. It is wise to check the pH of the medium and to adjust if necessary to pH 5·6.

Additions

(A) To 25 ml. stock substrate solution add 5 ml. reagent ethanol.
(B) Add $HgCl_2$(10^{-4}M) to stock substrate solution.
(C) Make up stock solution but omit glucose-1-phosphate and adenosine-5-phosphate.

Other Solutions

Gram's Iodine. Dilute with 10 times its volume of distilled water before use.

α-amylase. Make up a 0·5 per cent. solution in 0·004 M-acetate buffer (pH 5·5 to 6·0).

β-amylase. Make up a 0·5 per cent. solution in 0·004 M-acetate buffer (pH 4·0 to 5·7)

Method

(1) Cut 8-16μ sections on the cold microtome.
(2) Incubate sections, free-floating or mounted on coverslips, for 1–3 hours at 37°. If mounted sections are used these must not be allowed to dry in the air after removal from the instrument. If it is desired to demonstrate phosphorylase only, the single solution (A), containing ethanol, is used.
(3) Bring sections through 40 per cent. alcohol to water.
(4) Mount sections on slides (if not already mounted) and dry in an oven at 37°.
(5) Fix for 3 minutes in absolute alcohol and dry in air.
(6) Immerse in dilute Gram's iodine for 3 minutes.
(7) Mount in glycerine or in iodine-glycerine (1:5).

Result (Fig. 166)

A dark blue finely granular precipitate indicates sites of phosphorylase activity.

Further Tests

(1) After stage 5 incubate a section for 1–10 hours in α-amylase at 37°, wash in distilled water and apply dilute iodine solution as above. This section should show no coloured deposit.
(2) After stage 5 incubate a further section for 1–10 hours in β-amylase at 37° and proceed as above. This section should show only a mahogany-coloured deposit, indicating sites of transglycosylase activity.
(3) Sections incubated in stock substrate with $HgCl_2$ added should show only blue deposits, indicating phosphorylase.
(4) Native, pre-formed glycogen is detected in the section incubated without substrate or adenylic acid. In practice it is observed, on most occasions, to disappear during incubation.

Although sections stained with iodine last only for a few days or weeks at most, they can be restored quickly by removing the glycerine jelly and restaining in iodine. If permanent sections are required an alcoholic PAS procedure (Appendix 9, p. 831) should be applied.

APPENDIX 18

G-NADI REACTION FOR CYTOCHROME OXIDASE (after Moog)

(*fresh slices*)

Moog (1943) applied this method to whole chick embryos. It is equally applicable to thin unfixed tissue slices, or to thick sections.

Preparation of Reagents

(1) **"Nadi Reagent."** Just before use combine equal parts of 0·01 M dimethyl-*p*-phenylene diamine HCl and 0·01 M α-naphthol, each in 1 per cent. NaCl with 0·066 M phosphate buffer at pH 5·8.

(2) **Sodium Azide.** A 0·005 M solution of NaN_3 in physiological saline.

(3) **"Nadi Reagent" + Sodium Azide.** The Nadi reagent, as above, with 0·005 M sodium azide.

(4) **Phenylurethane.** A 0·003 M solution in physiological saline.

(5) **"Nadi Reagent" + Phenylurethane.** The Nadi reagent, as above, with 0·003 M phenylurethane.

Method

(1) Cut fresh tissue slices 100–150 μ thick into Ringer at pH 7·2.

(2) Incubate with "Nadi reagent" at 37° for 2–5 minutes.

(3) Incubate control sections, after washing in sodium azide and phenylurethane solutions respectively, in "Nadi reagent"/sodium azide (3) and in "Nadi reagent"/phenylurethane (5) for 2–5 minutes.

(4) Wash in physiological saline.

(5) Counterstain nuclei in carmalum (30 minutes) if desired.

(6) Mount in 5 per cent. potassium acetate and "ring" the coverslip with paraffin wax. Permanent preparations cannot be made.

Result

Sections incubated in "Nadi reagent" or in "Nadi reagent" with phenylurethane should show a blue or bluish-violet colour in areas of cytochrome oxidase activity. Sections incubated in the presence of sodium azide should be negative.

ADN METHOD FOR CYTOCHROME OXIDASE

(after Nachlas *et al.*)

(Cold microtome, fresh frozen sections ; mounted on slides or coverslips).

Preparation of the Medium

Mix the following reagents:

0·1 M-phosphate buffer (pH 7·4)	3·0 ml.
α-Naphthol (1 mg./ml.)	4·0 ml.
*Cytochrome c. (5 mg./ml.)	3·0 ml.
Catalase (30 μg./ml.)	1·0 ml.
Immediately before use add :	
ADN (2 mg./ml.)	4·0 ml.

* If known active tissues are being examined this (expensive) component of the medium may be omitted.

Method

(1) Incubate sections for 10–30 minutes at room temperature.
(2) Rinse briefly in 0·9 per cent. saline.
(3) Mount in glycerine jelly.

Result (Figs. 167 and 168)

Purple granules indicate sites of cytochrome oxidase activity.

Method for Preparation of ADN Hydrochloride

ADN (4-amino-1-N, N-dimethylnaphthylamine) hydrochloride is produced as follows:

Diazotization of Sulphanilic Acid

Dissolve 23 g. sodium sulphanilate and 7 g. sodium nitrite in 120 ml. water. Cool with ice and then pour, with stirring, into 17 ml. conc. H_2SO_4 containing 100 ml. of water cooled with ice. The diazonium compound is precipitated and the reaction is complete almost as soon as the last of the solution is added to the acid.

Coupling Reaction

Equimolar quantities of N, N-dimethyl-1-naphthylamine and diazotized sulphanilic acid are coupled by adding the suspended diazonium salt to the amine in twice its volume of glacial acetic acid. The insoluble azo compound is obtained. This is recrystallized from hot water to give blue-black needles melting at 266–8°.

Reduction

Dissolve 10 g. of the azo compound in 300 ml. hot 99·7 per cent. acetic acid to which 8 g. stannous chloride has been added. Heat on a water bath until the violet colour is completely discharged. Cool and make the mixture strongly alkaline with 5 N sodium hydroxide. Extract the free base with ether and dry over $CaCl_2$. The product is precipitated as the hydrochloride by passing dry HCl through the solution. M.P. 195–210°.

Recrystallization

Take up the solid hydrochloride in hot ethanol and treat with powdered charcoal. Filter and recrystallize from ethanol or ethanol-ether. A white product is obtained which sinters at 210° and melts with decomposition at 220°.

N-PHENYL PARAPHENYLENE DIAMINE METHOD FOR CYTOCHROME OXIDASE

(after Burstone)

(Fresh frozen, cold microtome sections ; mounted)

Preparation of Incubating Media

Place 10 mg. of the coupler * and 10 mg. N-phenyl-*p*-phenylene diamine in a 50-ml. Erlenmeyer flask. Add 0·5 ml. reagent ethanol, 35 ml. distilled

* As alternative couplers Burstone suggests *inter alia* the following compounds :
1-hydroxy-2-naphthoic acid ; 1-hydroxy-2-acetonaphthone ;
1-phenyl-3-(*m*-nitrobenzamidopyrazolone) ;
α·α′-terephthaloyl-bis(5-chloro-2, 4-dimethoxyacetanilide).

water and 15 ml. " Tris " buffer (0·2 M, pH 7·4). Shake and filter into a suitable container.

Method

(1) Incubate sections at room temperature for 15–60 minutes or longer.
(2) Transfer directly to 1 per cent. cobalt acetate in 10 per cent. formalin. Fix for 1 hour.
(3) Wash in water and mount in glycerine jelly.

Result (Fig. 169)

Reddish-brown to blue-black dyes are deposited at sites of cytochrome oxidase activity. These are finely particulate and stable.

METHOD FOR CATALASE (Nishiyama and Kobayashi)

(*Smears, fresh sections, cold acetone-fixed paraffin sections*)

Method

(1) Incubate sections in a mixture of equal parts of monoethyl hydrogen peroxide * and phosphate buffer (pH 6·8) for 10–15 minutes at 22°.
(2) Immerse in Schiff's reagent, 3–5 seconds.
(3) Rinse in sodium bisulphite solution.
(4) Wash, dehydrate, clear and mount in a permanent medium.

Result

A magenta colour was attributed by the authors to catalase activity (but see Chapter XVIII, p. 521).

ETHYL HYDROGEN PEROXIDE METHOD FOR " HYDROPEROXIDASE "

(after Watanabe)

(*Fresh frozen, cold microtome sections, mounted on slides or coverslips*)

Preparation of Solutions

Stabilizer. Dissolve 0·001 per cent. albumin, 0·001 per cent. glycogen and 0·001 per cent. urea in 0·1 M-veronal acetate buffer (pH 7·0).

Coupling Medium. 0·5 g. Fast blue B salt in 50 ml. of 2 per cent. pyridine in water.

Substrate. 1–2 per cent. ethyl hydrogen peroxide . 10 parts
Phenylhydrazine . . . . . 0·5 parts

Filter and add to 8 parts of the filtrate 1 part of phenylhydrazine and 1 part of the stabilizer solution.

The incubating medium should be freshly prepared before use, and adjusted to pH 7·0 with Na_2HPO_4.

Method

(1) Immerse sections for 1 minute in equal parts of carbon tetrachloride and light petroleum. Dry in air.
(2) Incubate for 5–10 minutes at 22° in the substrate medium.

* Prepared by method of Rieche and Hitz (1929).

(3) Wash in water.
(4) Treat with coupling medium for 4–6 minutes.
(5) Wash in water and fix in 10 per cent. neutral formalin for 10 minutes.
(6) Mount in glycerine jelly.

Result

Sites of " hydroperoxidase " activity are shown by the presence of a reddish-brown precipitate of diformazan (see Chapter XII, p. 339).

PREPARATION OF ETHYL HYDROGEN PEROXIDE (after Rieche and Hift)

Add 200 ml. 40 per cent. KOH, by dropping, into a mixture of 10 per cent. H_2O_2 (100 vol., 30 per cent.) in 100 ml. diethyl sulphate. Cool continuously with ice-salt mixture. After 30 minutes adjust the pH to 7·0 with 50 per cent. H_2SO_4.

Distil off the ethyl hydrogen peroxide (95–99°).

THE BENZIDINE REACTION FOR PEROXIDASE (after DeRobertis and Grasso)

(cold microtome, fresh frozen sections)

Method

(1) Cut sections, 20–40 μ thick, from fresh tissues, into 0·85 per cent. saline at 4°.

(2) Treat with 0·1 per cent. ammonium molybdate in 0·85 per cent. saline for 5 minutes.

(3) Immerse in saturated benzidine in 0·85 per cent. saline to which 1 drop of 20 vol. H_2O_2 per 2 ml. has been added immediately before. Move the sections in the liquid, with a glass rod, until a blue colour appears (30–40 seconds). Leave for a total of 2 minutes.

(4) Wash in 0·85 per cent. saline.

(5) Mount in glycerine jelly.

The preparations are not permanent.

Result

The presence of peroxidase is indicated by a blue precipitate.

AN IMPROVED BENZIDINE-PEROXIDASE REACTION (after van Duijn)

(Smears ; cold microtome, fresh frozen sections)

Solutions

(1) Saturated aqueous solution of benzidine. Dissolve 50 mg. in 200 ml. distilled water at 80°. Cool to room temperature and filter.

(2) 3 per cent. H_2O_2.

(3) Saturated ammonium chloride. Dissolve 40 g. NH_4Cl in 100 ml. of hot water and cool.

(4) 5 per cent. EDTA. Prepare a solution of ethylene diamine tetra-acetic acid and buffer to pH 6·0 with NaOH.

Incubating Medium

Add 1 ml. saturated ammonium chloride and 1 ml. EDTA solution to 9 ml. benzidine and add 1 drop of H_2O_2.

Method

(1) Incubate smears or sections for 5–10 minutes.
(2) Rinse briefly.
(3) Mount in PVA-Fructose medium.*

Result

Blue crystals indicate peroxidase activity. These are stable for some weeks.

α-NAPHTHOL REACTION FOR STABLE SUDANOPHILIA (after Ritter and Oleson)

(*Thin tissue slices; alkaline alcohol-formalin*)

Method

(1) Fix thin slices (1–4 mm.) of tissue in alcoholic alkaline formalin (95 per cent. alcohol, 90 ml. 40 per cent. formalin, 10 ml. 0·1 N NaOH, 1 ml.) for not less than 24 hours.
(2) Wash in running water for 30 minutes and stand in water for 2 hours.
(3) Immerse for 24 hours in α-naphthol-H_2O_2 solution, freshly prepared (α-naphthol 1 g., 40 per cent. alcohol 10 ml., 30 vol. H_2O_2 0·2 ml.).
(4) Rinse in running water.
(5) Stain for 3–24 hours in pyronin solution (pyronin 0·1 g., 40 per cent. alcohol 96 ml., aniline oil 4 ml.).
(6) Rinse in alcohol.
(7) Dehydrate in alcohol, clear in benzene and embed in paraffin wax.
(8) Cut sections 6–8 μ thick and mount on albuminized slides.
(9) Bring to water and counterstain nuclei if required.
(10) Dehydrate in alcohol, clear in xylene, mount in Canada balsam.

Result

Peroxidase granules (in the neutrophil leucocytes for instance) appear bright red.

(This method makes use of the increased basophilia of the granules after treatment with α-naphthol-H_2O_2.).

LEUCO-PATENT BLUE METHOD FOR PEROXIDASE (HÆMOGLOBIN) (after Dunn)

(*formalin; paraffin sections*)

Fix thin blocks (3–5 mm. thick) in 10 per cent. formalin buffered to pH 7·0. Embed in paraffin and section at 5–6 μ.

* Described by Spurr (1953), this consists of the following:

Polyvinyl alcohol (low viscosity grade)	18 g.
Cadmium iodide	34 g.
Fructose	8 g.
Distilled water	40 ml.

Wash the PVA with methanol, dry. Grind in a mortar. Dissolve the cadmium iodide and add the PVA with continuous stirring. Heat, while stirring, to 75° and add the fructose. After the foam has dispersed the medium is ready for use.

Preparation of Solutions

Prepare leueo-patent blue solution as follows : To Patent Blue V (C.I. No. 712) (1 g. in 100 ml. distilled water) add 10 g. powdered zine and 2·0 ml. glaeial aeetie acid. Boil until eolourless. Cool and filter. Before use add to 10 ml. of the stock solution 2 ml. glaeial aeetie aeid and 1 ml. 3 per eent. H_2O_2.

Prepare carmine alum solution as follows : Dissolve 1 g. carmine (alum lake) in 200 ml. of water with heat. Cool and add 0·2 g. salieylie aeid. Filter.

Method

(1) Bring paraffin seetions to water.
(2) Stain in freshly prepared working reagent, 3–5 minutes.
(3) Wash briefly in water.
(4) Counterstain in carmine alum, 30–60 seeonds (or in 0·1 per eent. safranin).
(5) Dehydrate in alcohols, clear in xylene and mount in DPX.

Result (Fig. 170)

Hæmoglobin stains dark blue-green. Background light pink, nuclei red (with safranin). Eosinophil and neutrophil granules stain dark blue.

THE DOPA-OXIDASE (TYROSINASE) REACTION (Becker *et al.*, 1935)

(*formalin*)

Method

(1) Fix tissues in 10 per cent. formalin for 1 hour at 22° (4–8 hours at 4°).
(2) Cut pieces 3–5 mm. thick and wash in running water, 3 minutes.
(3) Place in 0·0056 M-DOPA in 0·1 M phosphate buffer at pH 7·4. Ineubate for 1 hour at 37°.
(4) Change DOPA solution and ineubate 12–15 hours at 37°.
(5) Wash in running water.
(6) Fix in Bouin, 24 hours.
(7) Dehydrate in aleohols, clear in benzene or toluene, embed in paraffin.
(8) Cut sections 6–8 μ thiek. Counterstain as desired.
(9) Dehydrate, clear and mount in DPX or Canada balsam.

Result

Blackish-brown granules indieate the presenee of DOPA-oxidase (tyrosinase).

NAPHTHOIC HYDRAZIDE METHOD FOR MONOAMINE OXIDASE

(after Koelle and Valk; Eder)

(*Cold microtome, fresh frozen sections; mounted*)

Preparation of Stock Solutions

(1) 0·1 M-hydrazine hydroehloride.
(2) 40 per eent. Na_2SO_4 (w/v), adjusted to pH 8·6 with NaOH.
(3) 0·2 M-Na_2HPO_4 (buffer).

(4) 2-Hydroxy-3-naphthoic acid hydrazide (add to final medium).
(5) 0·1 M-*iso*nicotinyl-2-*iso*propyl hydrazine phosphate (Marsilid).
(6) 0·1 M-tryptamine hydrochloride.
(7) N.NaOH.

Preparation of Working Solutions

(1) Preincubating medium. 3·0 ml. water, 1·5 ml. hydrazine, 3·0 ml. buffer, 7·5 ml. Na_2SO_4 (pH should be 7·6).

(2) Control pre-incubating medium. As above with 0·15 ml. Marsilid.

(3) Rinse. 4·5 ml. water, 3·0 ml. buffer and 7·5 ml. Na_2SO_4.

(4) Incubating medium. 4·35 ml. water, 0·15 ml. NaOH, 3·0 ml. buffer, 7·5 ml. Na_2SO_4. Heat to 80–90° and saturate with naphthoic acid hydrazide. Cool, filter and add 1·0 ml. tryptamine. Saturate with O_2 immediately before use.

(5) Control incubating medium. Prepare as (4) but add, finally, 0·15 ml. Marsilid.

(6) Developing medium. 300 mg. Fast blue B salt, dissolved in 10 ml. water with 5 ml. phosphate buffer, pH 7·4. Filter and use at once.

Method

(1) Immerse sections in pre-incubating medium for 1 hour at 22°.
(2) Rinse in buffered sodium sulphate medium.
(3) Blot, dry, and immerse in incubating medium for 2 hours at 37°. During incubation a stream of O_2 must be passed through the medium.
(4) Rinse in distilled water.
(5) Develop in Fast blue B solution for 3 minutes at room temperature.
(6) Rinse in distilled water.
(7) Fix in 10 per cent. formalin for 1 hour.
(8) Mount in glycerine jelly.

Result (Fig. 172)

A purplish-blue colour indicates sites of MAO activity. Control sections preincubated in Marsilid and incubated in the presence of this inhibitor should show no colour. Control sections incubated in the absence of substrate should likewise show no colour.

TETRAZOLIUM METHOD FOR MONOAMINE OXIDASE

(after Glenner *et al.*)

(Cold microtome, fresh frozen sections ; mounted)

Preparation of Incubating Medium

Tryptamine hydrochloride	25 mg.
Sodium sulphate	4 mg.
Nitro-blue tetrazolium	5 mg.
0·1 M-Phosphate buffer (pH 7·6)	5 ml.
Distilled water	15 ml.

Method

(1) Incubate 20–40 μ fresh sections, mounted on coverslips, for 30–45 minutes at 37°.

(2) Wash in running water for 2 minutes.
(3) Fix in 10 per cent. neutral formalin for 24 hours.
(4) Mount in glycerine jelly.*

Result

Blue formazan deposits indicate sites of MAO activity.

* Purple and red colours may also indicate activity. These are removed if, as the original authors advise, sections are dehydrated, cleared and mounted in Permount. I do not advise this procedure.

APPENDIX 20

METHODS FOR DPN AND TPN DIAPHORASES USING MTT

(Scarpelli, Hess and Pearse, 1958)

(*Fresh frozen, cold microtome sections, mounted on slides or coverslips*)

Stock Solution

MTT, 1 mg./ml.	2·5 ml.
Cobaltous chloride, 0·5 M	0·3 ml.
0·06 M-Phosphate buffer (pH 8·2)	2·5 ml.
Distilled water	4·1 ml.
Polyvinyl pyrrolidone	0·75 ml.

The phosphate buffer and the cobaltous chloride should be mixed and the precipitate formed should be removed by filtration prior to addition of the remaining components. Since the addition of the cobalt salt results in a marked lowering of the pH, the solution must be adjusted to pH 7·2 with 0·2 M-Tris buffer (Stock, pH 10·4), using a glass electrode. Keeps for 3–4 weeks at 0–4°.

Alternatively, the use of phosphate buffer can be avoided entirely and 0·2 M-Tris (pH 8·0) substituted for it. Adjustment of the final pH is still necessary. Of the two alternatives the Tris modification is recommended for routine use.

DPN Diaphorase and TPN Diaphorase Media

Add to 1 ml. of the stock solution 6 mg. of DPNH or TPNH. Adjust pH to 7.0 with one or two drops of Tris (pH 8·5) buffer. The final concentration of reduced pyridine nucleotide is 1×10^{-2} M. These solutions remain stable for $1\frac{1}{2}$ to 2 hours. Increased activity can be demonstrated in most tissues by the addition to the above medium of sufficient amobarbital (amytal) to make a final concentration of 1×10^{-2} M. The addition of amytal (22·6 mg.) to 10 ml. of incubating medium will necessitate further adjustment of the pH to the required pH 7·2.

Method

(1) Place 0·1 to 0·2 ml. incubating medium on a freshly cut section mounted on a coverslip.

(2) Incubate for 5–30 minutes at 37° in air.

(3) If phosphate buffer has been used, rinse in 1 per cent. HCl.

(4) Fix in 10 per cent. formol-calcium or formol-saline for 5–30 minutes.

(5) Rinse in distilled water.

(6) Mount in glycerine jelly.

Nuclei may be counterstained, if required, in dilute (0·5 per cent.) chloroform-washed aqueous methyl green, or in carmalum (Mayer's) diluted 1 : 10 with distilled water.

Result (Fig. 190)

Intramitochondrial deposits of black cobalt formazan indicate sites of DPN or TPN diaphorase.

METHOD FOR DPN DIAPHORASE USING NITRO-BT
(after Nachlas, Walker and Seligman, 1958)

(*Fresh frozen, cold microtome sections, mounted on slides or coverslips*)

Preparation of Incubating Medium

Sodium lactate (0·5 M)	0·6 ml.
Lactic dehydrogenase (1·5 per cent. aqueous)	0·2 ml.
DPN (5 mg./ml.)	0·3 ml.
Nitro-BT (5 mg./ml.)	0·3 ml.
Phosphate buffer pH 7·4 (0·2 M)	1·0 ml.
Distilled water	0·6 ml.

The DPN and the dehydrogenase solutions are made up without adjustment of pH and stored at — 20°. The lactate and Nitro-BT solutions are adjusted to pH 7·4 and stored at 6° for up to 3 months.

Method

(1) Mount sections on coverslips (drying in a stream of air, advised by the original authors, is not recommended).
(2) Incubate at 22° for 5–30 minutes in air.
(3) Rinse briefly in saline.
(4) Fix in 10 per cent. formol-saline for 10 minutes.
(5) Mount in glycerine jelly.

Result

The blue diformazan deposits indicate the localization of DPN diaphorase. Any red colour present may be ignored. It can be (partially) removed by rinsing in 10–15 per cent. alcohol between stages 4 and 5.

METHOD FOR TPN DIAPHORASE USING NITRO-BT
(after Nachlas, Walker and Seligman, 1958)

(*Fresh frozen, cold microtome sections, mounted on coverslips*)

Preparation of Incubating Medium

Sodium DL-*iso*citrate (1·1 M)	0·6 ml.
Sodium L-malate (2·5 M)	0·5 ml.
Manganese chloride (0·005 M)	0·3 ml.
TPN (5 mg./ml.)	0·2 ml.
Nitro-BT (5 mg./ml.)	0·3 ml.
Veronal acetate buffer (0·05 M), pH 7·4	1·1 ml.

Stock solutions of substrates are adjusted to pH 7·4 at the time of preparation as is the solution of Nitro-BT. These are stored at 6°. The TPN solution (unbuffered) is kept at — 20°.

Method

Details are as given in the method for DPN diaphorase, above.

Result

As for DPN diaphorase.

METHOD FOR SUCCINATE DEHYDROGENASE USING NITRO-BT (after Nachlas *et al.*, 1957)

(*Fresh frozen, cold microtome sections, mounted on coverslips*)

Preparation of Substrate Solutions

Stock Buffered Succinate. Combine equal volumes of 0·2 M-phosphate buffer (pH 7·6) and 0·2 M-sodium succinate.

Incubating Medium. Add 10 ml. stock succinate solution to 10 ml. aqueous solution of Nitro-BT (1 mg./ml.).

Method

(1) Incubate sections for 5–20 minutes at 37° in air.
(2) Wash in saline.
(3) Fix in 10 per cent. formol-saline for 10 minutes.
(4) Rinse in 15 per cent. alcohol for 5 minutes.
(5) Mount in glycerine jelly.

Result (Plate VIIB)

Blue diformazan deposits indicate sites of succinate dehydrogenase activity.

METHODS FOR SUCCINATE DEHYDROGENASE USING MTT (Modified from Pearse, 1957)

(*Fresh frozen, cold microtome sections, mounted on coverslips*)

Preparation of Incubating Media

Two alternative media are given. The first is recommended for critical studies. The second is easier to prepare.

(1) Add to 2·5 ml. 0·06 M phosphate buffer (pH 7·4) 0·3 ml. cobaltous chloride (0·5 M). Filter.

Add to the filtrate 2·5 ml. MTT solution (1 mg./ml.) and 3·0 ml. sodium succinate (0·57 g./10 ml.). Adjust the pH to 7·0 with stock Tris buffer. Add distilled water to 10 ml. and 0·75 g. polyvinylpyrrolidone.

(2) Add to 2·5 ml. 0·2 M Tris buffer (pH 7·1 to 7·2) 0·3 ml. cobaltous chloride (0·5 M), 2·5 ml. MTT (1 mg./ml.), 3·0 ml. sodium succinate (0·54 g./10 ml.) and 1·7 ml. distilled water. Dissolve 0·75 g. polyvinylpyrrolidone in this solution.

Method

(1) Place 0·1 to 0·2 ml. of incubating medium on each section.
(2) Incubate at 37° for 5–30 minutes, in air.
(3) If phosphate buffer has been used rinse sections briefly in 1 per cent. HCl.
(4) Fix in 10 per cent. formol-calcium or formol-saline for 10 minutes.
(5) Mount in glycerine jelly containing 0·5 M-cobaltous acetate.

Nuclei may be counterstained if necessary.

Result (Figs. 191–193 ; Plate VIIC)

Black cobalt formazan deposits indicate sites of succinate dehdrogenase activity.

METHODS FOR DEMONSTRATION OF DPN AND TPN-LINKED DEHYDROGENASES (Hess, Scarpelli and Pearse, 1958)

The various incubating media are based on the original cobalt-formazan technique (Pearse, 1957) and on the techniques of Nachlas, Walker and Seligman (1958a and b).

Preparation of Incubating Media

These should be freshly prepared before use. The composition of the standard medium is as follows* :

Substrate (1·0 M)	0·1 ml.
DPN or TPN (1·0 to 0·1 M)†	0·1 ml.
Respiratory inhibitor (amytal, azide or cyanide)‡ (0·1 M)	0·1 ml.
Magnesium chloride (0·05 M)	0·1 ml.
Buffer (0·06 M-phosphate or 0·2 M-Tris) (pH 6·8–7·0)	0·25 ml.
Tetrazolium salt (MTT or Nitro-BT) (1 mg./ml.)	0·25 ml.
Distilled water to make	1·0 ml.
Polyvinylpyrrolidone (M.W. 11,000)	75 mg.

For demonstrating the different dehydrogenase systems the components of the above standard medium are varied as follows :

A. Using MTT

(1) **β-Hydroxybutyrate dehydrogenase.** Substrate DL-β-hydroxybutyric acid sodium salt ; DPN ; amytal or sodium azide, phosphate buffer. Mix 0·05 ml. of 0·5 M-cobaltous chloride ($CoCl_2.6H_2O$) with the buffer and filter. Add the other constituents of the medium to this filtrate and adjust the final pH to 7·0 with stock Tris buffer. Incubation time should not exceed 15 minutes.

(2) **Glucose-6-Phosphate dehydrogenase.** Substrate, glucopyranose-6-phosphate disodium (or calcium) salt ; TPN ; sodium azide or amytal ; Tris buffer ; 0·05 ml. of 0·5 M-$CoCl_2$; sodium fluoride (0·01 M) 0·05 ml.

(3) **6-Phosphogluconate dehydrogenase.** Substrate, 6-phosphogluconic acid barium salt (0·01 M) ; TPN ; sodium azide or amytal ; Tris buffer ; 0·05 ml. of 0·5 M-$CoCl_2$.

B. Using Nitro-BT

(1) ***Iso*citrate dehydrogenase.** Substrate, DL-*iso*citric acid, adjusted to pH 7·0 with Tris ; DPN or TPN ; sodium cyanide ; phosphate buffer.

(2) **Malate dehydrogenase.** Substrate, sodium-L-malate or L-malic acid adjusted to pH 7.0 with Tris ; DPN or TPN ; sodium cyanide ; phosphate buffer.

(3) **Glutamate dehydrogenase.** Substrate, sodium-L-glutamate monohydrate, DPN or TPN ; sodium cyanide ; phosphate buffer.

(4) **α-Glycerophosphate dehydrogenase.** Substrate, sodium DL-α-glycero-

* The individual constituents of the media, including the substrate but excluding the coenzyme, can be buffered to pH 7·0 and stored at 0–4°.

† DPN and TPN can be stored (at slightly acid pH levels) in solution at — 20°. For most purposes a stock concentration of 0·1 M and a final concentration of 0·01 M is adequate.

‡ The cyanide solution must be adjusted to pH 7·2 with N-HCl.

phosphate (neutralized with 0·1 M-HCl) ; DPN ; sodium cyanide ; Tris buffer.

(5) **Alcohol dehydrogenase.** Substrate, ethanol ; DPN ; sodium cyanide ; phosphate buffer.

(6) **Lactate dehydrogenase.** Substrate, sodium DL-lactate ; DPN ; sodium cyanide ; phosphate buffer.

Method

(1) Cover the section with 0·1 to 0·2 ml. incubating medium and incubate for 5–30 minutes at 37°, in air.

(2) If cobalt phosphate precipitates are present rinse the sections briefly (30 seconds) in 1 per cent. HCl.

(3) Fix in 10 per cent. formol-saline or formol-calcium for 10 minutes.

(4) Mount in glycerine jelly containing 0·5 M cobaltous acetate, or in plain glycerine jelly.

Nuclei may be counterstained as necessary ; methods are described above.

Result (Figs. 187 and 188)

In each case the black cobalt-formazan deposits or deep purple diformazan deposits in the mitochondria indicate activity of the respective enzyme systems. With MTT there is sometimes a diffuse grey extramitochondrial staining. With Nitro-BT a red product may be present. This can be reduced to a low level by treatment with 10–15 per cent. alcohol before mounting in glycerine jelly.

APPENDIX 21

METHOD FOR LEUCINE AMINOPEPTIDASE
(Nachlas, Crawford and Seligman, 1957)

(Fresh frozen, cold microtome sections, mounted; freeze-dried paraffin-embedded sections)

Preparation of the Substrate Solution

Stock Substrate. Dissolve 8 mg. per ml. of L-leucyl-β-naphthylamide (I) or L-leucyl-4-methoxy-β-naphthylamide (II)* in distilled water. These solutions can be stored at 0–4° for several months.

Incubating Medium

Stock substrate solution	1 ml.
Acetate buffer (0·1 M, pH 6·5)	10 ml.
Sodium chloride (0·85 per cent.)	8 ml.
Potassium cyanide (2×10^{-2} M)	1 ml.
Fast blue B salt	10 mg.

Method

(1) Incubate sections for 15 minutes to 2 hours at 37°.
(2) Rinse in saline for 2 minutes.
(3) Immerse in 0·1 M-cupric sulphate for 2 minutes.
(4) Rinse again in saline.
(5) Dehydrate in graded alcohols, clear in xylene and mount in Canada balsam. Alternatively sections may be mounted without dehydration in glycerine jelly or polyvinyl pyrrolidone medium.

Result

A purplish blue colour (I) or a bright red colour (II) indicates the site of leucine aminopeptidase activity.

METHOD FOR CATHEPSIN C. USING INDOXYL ACETATES
(Hess and Pearse, 1958)

(Cold formalin-fixed frozen sections, free-floating)

Method

(1) Incubate sections for 1 hour at 37° in 10^{-5} M–E600 (diethyl-*p*-nitrophenyl phosphate). This procedure inactivates all B-type esterases present in the tissues.

(2) Incubate a control section for 30 minutes to 16 hours in the standard indoxyl esterase medium (Appendix 16, p. 000).

(3) Pre-incubate test sections for 1 hour at 37° in 0·1 M-veronal acetate buffer (pH 5·3) containing one of the following activators :

Ascorbic acid (2×10^{-3} M)
Cysteine (1×10^{-3} M)
Glutathione (1×10^{-3} M)
Tween 60 (10^{-3} g./100 ml.)
Tween 80 (10^{-3} g./100 ml.)
Phenylmercuric chloride (1×10^{-4} M)

* Owing to the low solubility of this substrate it should first be dissolved in a few drops of methanol and then diluted with water to the required volume.

or inhibitors :

Lead nitrate (1×10^{-3} M)
β-phenylpropionic acid (1×10^{-2} M)

(4) Incubate sections, for the same period as the controls, in the standard indoxyl esterase medium containing the activator or inhibitor used for pre-incubation. (Cysteine and β-phenylpropionic acid are a useful pair for routine use. Activation, by pre-incubation only, with trypsin is also useful).

(5) Wash in distilled water.

(6) Mount in glycerine jelly.

Result

Any cell, containing indigo in the control section, which contains more indigo after incubation with the activators and less after the use of inhibitors can be considered to exhibit esterase activity of the type associated with cathepsin C.

METHOD FOR ARYLSULPHATASE
(Rutenburg, Cohen and Seligman, 1952)

(Cold formalin-fixed, frozen sections; cold microtome sections, mounted)

Preparation of Substrate Solution

Dissolve 25 mg. potassium 6-benzoyl-2-naphthyl sulphate in 80 ml. hot 0·85 per cent. sodium chloride solution. Add 20 ml. 0·5 M-acetate buffer (pH 6·1). This solution is stable and can be stored for at least 15 days. Before use this solution is made hypertonic by the addition of 2·6 g. solid sodium chloride to each 100 ml.

Method

(1) Pretreat sections, for 2–5 minutes in each, in successive solutions of sodium chloride (0·85, 1·0 and 2·0 per cent.).

(2) Incubate for 2–8 hours at 37° in the substrate solution.

(3) Wash twice with cold saline (fresh tissues) or water (formalin-fixed tissues).

(4) Transfer to an ice-cold freshly prepared solution of Fast blue B salt (1 mg./ml.) in 0·05 M-phosphate buffer (pH 7·6). Agitate gently for 5 minutes.

(5) Wash three times in cold 0·85 per cent. saline and then in water.

(6) Mount in glycerine jelly.

Result

A blue or purplish-blue colour indicates sites of strong arylsulphatase activity. A red or pink colour may indicate weaker activity or diffusion of the final product with solution in lipids.

METHOD FOR CARBONIC ANHYDRASE
(Hausler, 1958: after Kurata)

(Cold microtome, fresh sections, post-fixed in cold acetone; free-floating)

Preparation of Incubating Medium

Solution A. To 1·0 ml. 0·1 M $CoSO_4$ add 6·0 ml. 0·05 M-H_2SO_4. (This solution is stable.)

Solution B. Dissolve 1·0 g. $NaHCO_3$ in 50 ml. 0·1 M-Na_2SO_4. (Freshly prepared before use.)

Before incubation pour solution B into solution A in order to avoid a momentary high concentration of cobalt ions.

Method

(1) Post-fix sections in cold absolute acetone (0–4°) for 1 hour.

(2) Transfer directly into incubating medium at 18–20°. Incubate for $1\frac{1}{2}$ to 2 hours. It is important to keep the sections floating on the surface of the medium.

(3) Wash in distilled water for 2 minutes.

(4) Transfer to dilute $(NH_4)_2S$ solution for 1 minute.

(5) Wash in tap water.

(6) Mount in glycerine jelly.

Result

A positive result is indicated by black or brown deposits of cobalt sulphide. Control sections incubated in the presence of 4×10^{-3} M-Diamox (sodium salt) should show no staining.

APPENDIX 22

RIBONUCLEASE EXTRACTIONS

(Formalin, Bouin, Carnoy, etc. ; paraffin sections ; cold microtome sections, post-fixed in acetic-ethanol)

Crystalline ribonuclease can be made by the McDonald (1948) modification of the Kunitz (1940) method, from fresh beef pancreas. It is obtainable commercially in the United States and also in this country.

Method

(1) Bring paraffin sections to water.

(2) Incubate for 1 hour at 37° in a solution of the enzyme (0·5 to 1 mg./ml.)* in glass-distilled water.

(3) Wash in running water.

(4) Stain the treated and a control section by whatever method is desired. (For choice of methods for staining the sections see text, Chapter VIII).

Result (see Figs. 68 and 69)

Basophilic material removed by this treatment is to be regarded as ribonucleic acid.

DEOXYRIBONUCLEASE EXTRACTIONS

(Zenker, Carnoy, etc.; paraffin sections. Acetic-ethanol, cold microtome sections)

Crystalline deoxyribonuclease is available from several sources in the United States and also in this country. Streptodornase-Streptokinase (Lederle) can be used instead.

Method

(1) Bring paraffin sections to water.

(2) Treat sections for 1–24 hours at 37° with streptodornase (1,000 units/ml.) in 0·025 M-Veronal buffer at pH 7·5 containing 0·003 M-$MgSO_4$.† Renew enzyme solution occasionally. Take care not to shake the enzyme solution. This hastens its destruction.

(3) Wash in water.

(4) Dehydrate in alcohol and pass to alcohol/ether.

(5) Cover with a thin film of 1 per cent. celloidin.

(6) Perform the standard Feulgen reaction (p. 822).

Result

Control slides show staining of nuclear chromatin, treated slides should not do so.

* As low as 0·1 m.g./ml. is often sufficient.

† The relationship of the concentration of enzyme to Mg^{2+} ions is important. The crystalline enzyme should be used at 1 mg./ml. with 0·0025 M-Mg^{2+} ions. Incubation up to 48 hours may be necessary at times.

HYALURONIDASE EXTRACTIONS

(Formalin, alcohol, etc.; paraffin sections. Acetic ethanol, cold microtome sections)

Two commercial preparations of testis hyaluronidase which I have used give satisfactory results. These are Hyalase (Benger) and Rondase (Evans Medical Supplies Ltd.).

Method

(1) Bring paraffin sections to water.
(2) Incubate for 3 hours at 37° in a solution of either of the above-mentioned preparations (1 mg./ml.) in 0·85 per cent. saline.
(3) Wash thoroughly in distilled water.
(4) Stain in $\frac{1}{2}$ per cent. aqueous toluidine blue, 20 minutes.
(5) Rinse in distilled water, mount in water and compare with a control section incubated at 37° for 3 hours in saline alone, and stained as above.

Result (see Plate VIIIB)

For interpretation of hyaluronidase-lability see text, Chapter XXII.

TRYPSIN EXTRACTIONS

(Carnoy; smears or paraffin sections)

Method

(1) Bring sections to water.
(2) Incubate for 15–60 minutes at 37° in 0·05 M phosphate buffer at pH 8·9, containing 0·1 mg./ml. purified trypsin. Incubate control section in buffer without enzyme. As an alternative 0·2 mg./ml. in 0·05 M-borate buffer can be employed.
(3) Wash both sections in water.
(4) Treat by whatever staining method is considered desirable (see text, Chapters V, VIII and IX).

Result

Histone and non-histone proteins alike are degraded, and removed by the subsequent washing in water.

PEPSIN EXTRACTIONS

(Smears or paraffin sections after Carnoy fixation)

Method

(1) Bring sections to water.
(2) Incubate for 2–3 hours at 37° in 0·02 N HCl at pH 1·6, containing 2 mg./ml. crystalline pepsin. Incubate control section in acid only.
(3) Wash both sections in water.
(4) Treat by whatever staining method is considered desirable (see text, Chapter XXII).

Result

Kaufmann *et al.* (1950) regard the shrinkage observed in tissues after treatment with pepsin as due to removal, primarily, of tryptophan-containing proteins.

PECTINASE DIGESTION (after McManus and Cason)

(Acetone; paraffin sections)

Pectinase Solutions

McManus advises solutions of Pectinase (Nutritional Biochemicals Corp.) or Pectinol O (Rohm and Hass). A commercial preparation, containing pectinesterase and polygalacturonase, available in this country, is Pectozyme (Norman Evans and Rais Ltd.).

Dissolve 0·8 g. of the enzyme in 100 ml. of acetate buffer at pH 4·2. The buffer solution alone should be used for control sections.

Method

(1) Bring sections to distilled water via light petroleum and absolute acetone.

(2) Incubate for up to 48 hours in the enzyme solution.

(3) Wash thoroughly in tap water.

(4) Stain buffer control and test sections by the selected procedure (usually the PAS method).

Result

PAS-positive materials are removed from animal tissues, presumably by the polygalacturonase present in pectinase. This action is reversibly blocked by previous acetylation of the sections (see Appendix 5 for details of acetylation).

APPENDIX 23

MASSON-FONTANA METHOD FOR MELANIN (WITH HEXAMINE-SILVER VARIANT)

(various, not chromate; paraffin sections)

Silver Solutions

(1) Fontana

To 20 ml. of 10 per cent. $AgNO_3$ add strong ammonia drop by drop until only a few granules of the first formed precipitate remain. Add 20 ml. of distilled water and allow to settle for 24 hours. Decant into a dark bottle. Filter each jar before use and do not use for more than a few sections. The solution keeps for about a month.

(2) Hexamine-silver (Gomori)

To 100 ml. of 3 per cent. hexamine add 5 ml. of 5 per cent. $AgNO_3$. A precipitate forms and redissolves. Add 5 ml. of borate buffer of approximately pH 8. (Add a drop of phenolphthalein to 3 per cent. boric acid and titrate with N NaOH until just pink.) Make up to 200 ml. with distilled water.

This M/5 borate buffer tends to crystallize. Burtner and Lillie (1949) recommend the substitution of boric acid-borate buffer (pH 7·8), 25 ml. per 100 ml. hexamine silver solution (Appendix 1).

Method

(1) Bring paraffin sections to water.
(2) Treat with Gram's iodine solution for 10 minutes and wash in running distilled water.
(3) Leave sections for 18–24 hours in one of the two silver solutions given above, in the dark in a covered jar.
(4) Rinse in distilled water.
(5) Fix in 5 per cent. sodium thiosulphate, 1–2 minutes.
(6) Counterstain nuclei with 1 per cent. carbol safranin,* 10 minutes.
(7) Differentiate in 70 per cent. alcohol.
(8) Dehydrate rapidly in alcohol, clear in xylene and mount in Canada balsam.

Result (Fig. 200)

Melanin granules appear black, nuclei red, keratin orange.

THE FERROUS IRON TECHNIQUE FOR MELANIN (Lillie, 1957)

(Formalin, Carnoy, etc., not chromate; paraffin section)

Method

(1) Bring paraffin sections to water.
(2) Immerse for 1 hour in 2·5 per cent. ferrous sulphate ($FeSO_4.7H_2O$)

* Melt 0·5 g. phenol in a dry flask, add 0·2 g. safranin and mix well. Dissolve the sludge in 50 ml. starch-dextrin solution. (Grind 0·25 g. starch with 0·25 g. dextrin and add 50 ml. of distilled water while continuing to grind. Heat to 80°, cool and filter.)

(3) Wash for 20 minutes in 4 changes of distilled water.

(4) Immerse for 30 minutes in 1 per cent. potassium ferricyanide in 1 per cent. acetic acid.

(5) Wash in 1 per cent. acetic acid.

(6) Counterstain, if desired, in Van Giesons picric-acid fuchsin.

(7) Dehydrate, clear and mount in synthetic resin.

Result

Melanins, dark green; background faint green or colourless (unless counterstained).

BLEACHING METHODS FOR MELANIN

(1) Permanganate

Treat sections with 0·1 per cent. $KMnO_4$ for 12–24 hours, rinse in water and treat with 1 per cent. oxalic acid for 1 minute.

(2) Chlorate

Add a small quantity of $KClO_3$ to a Coplin jar containing 40–50 ml. of 50 per cent. alcohol. Add a few drops of conc. HCl. Immerse sections for 24–48 hours. Wash.

(3) Bromine

Treat sections with 1 per cent. bromine water for 12–24 hours.

(4) Chromic Acid

Treat sections with a mixture of equal parts of 1 per cent. chromic acid and 5 per cent. $CaCl_2$ for 8–12 hours.

(5) Peroxide

Treat sections with 30 vol. (10 per cent.) hydrogen peroxide for 24–48 hours.

(6) Peracetic acid

Treat sections with 40 per cent. peracetic acid for 2–16 hours.

Of all these methods the last is probably the best. Most melanins in paraffin sections are bleached within 16 hours and if the sections are affixed with egg albumin to the slides no harm is done to the tissues. Other pigments (e.g. lipofuscins) tend to resist for longer than 16 hours. The permanganate and chromic acid routines bleach all pigments and the rate of bleaching does not allow any differentiation to be made between one pigment and another.

CHROMAFFIN REACTION FOR ADRENALIN AND NORADRENALIN (Hillarp and Hökfelt)

(*Fresh tissues*)

Method

(1) Treat thin slices of tissue with a solution containing 10 vols. 5 per cent. $K_2Cr_2O_7$ and 1 vol. 5 per cent. K_2CrO_4 (pH 5·6). Leave for 16 hours at room temperature.

(2) Wash in distilled water for 30–60 minutes ; 3 changes.

(3) Mount in glycerine jelly after transferring sections to clean slides.
(4) Alternatively sections can be dehydrated, cleared and mounted in Canada balsam.

Result

Adrenalin-containing cells stain dark brown; noradrenalin usually stains more lightly (yellow or yellowish-brown).

IODATE METHOD FOR NORADRENALIN (after Hillarp and Hökfelt, 1955)

(*Fresh tissues*)

Method

(1) Transfer thin (0·5 mm.) slices of adrenal gland to 10 per cent. aqueous potassium iodate. Leave for 16 hours at room temperature.
(2) Transfer to 10 per cent. formalin for 2 hours.
(3) Cut frozen sections 10–20μ thick.
(4) Wash well in distilled water.
(5) Counterstain nuclei if required.
(7) Mount sections on slides; dehydrate, clear and mount finally in Canada balsam.

Result (Fig. 202)

Cells which contained noradrenalin show a diffuse brown staining which, in counterstained preparations, affects nuclei as well as cytoplasm.

MODIFIED GIEMSA METHOD FOR ADRENOCHROME (CHROMAFFIN) (after Sevki)

(*Müller, Orth, Zenker etc.; paraffin sections*)

The original author fixed his material in formalin and his results differed considerably from those which are obtained with chromate-fixed material. Whereas after formalin fixation the granules of the adrenal medullary cells are acidophilic and stain rose-red with Giemsa, after chromate fixation they are moderately basophilic and take up some of the azure component.

Method

(1) Bring sections to distilled water and leave for 30–60 minutes.
(2) Stain in dilute Giemsa (1 drop to 1 ml. distilled water) for 24 hours.
(3) Rinse in distilled water.
(4) Differentiate quickly in 90 per cent. alcohol.
(5) Pass through absolute alcohol to xylene and mount in Canada balsam.

Result

Adrenochrome-containing (chromaffin) granules stain greenish-yellow, nuclei dark blue, cytoplasm of other cells, pink to red. (Similar results can be obtained by brief staining with methylene blue except that the cytoplasm of other cells is unstained.)

Chromaffin granules have moderately strong reducing properties and they will progressively darken if kept in ammoniacal silver solutions. Ferricyanide is quickly reduced and Schmorl's test (see p. 925) is therefore positive.

FLUORESCENCE METHOD FOR NORADRENALIN (Eränkö, 1955)

(*Fresh tissues*)

Method

(1) Cut fresh frozen sections at 50μ by means of the cold knife procedure or with a cold microtome.

(2) Allow sections to thaw on the surface of a solution containg 1 vol. 40 per cent. formaldehyde, 5 vols. 2 per cent. $CaCl_2$ and 4 vols. distilled water.

(3) After 2–6 hours rinse the sections in distilled water, mount on slides and cover with glycerol and a coverslip.

Result (Fig. 203)

With the fluorescence microscope cells which contained noradrenalin show a strong yellowish-green fluorescence. The nuclei do not fluoresce.

DIAZO METHOD FOR ARGENTAFFIN CELL GRANULES

(*Formalin: paraffin sections. Avoid mercury*)

Method

(1) Bring sections to water.

(2) Treat for 30 seconds with a dilute (1 mg./ml.) solution of the stable diazotate of 5-nitroanisidine (Fast Red salt B, I.C.I. Ltd.), also known as Echtrotsalz B, in 0·1 M veronal acetate buffer at pH 9·2.

(3) Wash thoroughly in running water.

(4) Stain nuclei with Mayer's hæmalum, 6–10 minutes.

(5) Wash in running water for 30 minutes.

(6) Dehydrate in alcohol, clear in xylene and mount in DPX.

Result (Plate VIIIc)

Argentaffin (E.C) cell granules, fiery orange red. Nuclei, dark blue. Cytoplasmic structures, yellow.

(Any one of a large variety of stable diazotates can be used; most satisfactory results are obtained with those giving reddish azo dyes. The colours given with granules in carcinoid tumours are more usually brownish-red.)

ACID DIAZONIUM REACTION FOR AROMATIC AMINES AND PHENOLS

(*Formalin; paraffin sections*)

Method

(1) Bring sections to water.

(2) Immerse in 0·5 M acetate buffer (pH 5·2) containing 1 mg./ml. of diazotized *p*-nitroaniline. Leave for 20–40 minutes.

(3) Wash in running water.

(4) Counterstain nuclei with Mayer's hæmalum, 3 minutes.

(5) Wash in running water for 30 minutes.

(6) Dehydrate in alcohol, clear in xylene and mount in DPX.

Result

Argentaffin granules in the enterochromaffin cells or in carcinoid tumours stain orange-red. Other tissue components which possibly contain aromatic amines (e.g. pseudomelanin in the intestine) are coloured to a lesser extent.

THE ALKALINE THIOINDOXYL METHOD FOR QUINONEIMINES

(*Formalin; paraffin sections*)

Method

(1) Bring sections to water.

(2) Oxidize for 10 minutes in either 5 per cent. iodine in 95 per cent. ethanol, or in 2·5 per cent. aqueous sodium iodate at 56°.

(3) After iodine, bring to water and treat for 2 minutes with 5 per cent. aqueous sodium thiosulphate.

(4) Rinse briefly in water.

(5) Incubate for 30 minutes at 56° (or for 2–4 hours at 37°) in the alkaline thioindoxyl solution.*

(6) Wash in water and counterstain nuclei, if required, with hæmalum.

(7) Dehydrate, clear and mount in a suitable synthetic resin.

Result

E.C. granules appear orange-red to reddish-brown.

STABLE DIAZOTATES

Stable diazotates are produced in four different ways to give products with very different stabilities.

(1) The diazonium salt is warmed with alkali to form the anti-diazotate. This is very stable but easily convertible by the addition of mineral acid to the free diazoic acid. The latter breaks down rapidly to a nitrosamine.

$$C_6H_5.N \underset{NOK}{\overset{\|}{}} \longrightarrow C_6H_5N \underset{NOH}{\overset{\|}{}} \longrightarrow C_6H_5NHNO$$

Diazoic acid Phenyl nitrosamine

The nitrosamines are yellow compounds and they are partly responsible for the staining of the background structures in tissue sections exposed to diazotates in solution.

(2) The diazonium salt is coupled with sarcosine or methyl taurine, to give a very stable compound which releases the free diazonium salt on acidification.

(3) By the addition of naphthalene disulphonic acids to diazonium salts stable derivatives are formed. In this way Manheimer and Seligman (1949) produced a stable diazotate of α-naphthylamine for use in their modification of the alkaline phosphatase technique of Menten *et al.* (1944).

(4) By the addition of zinc salts diazo double salts are produced. These are relatively stable and they do not require acidification for activation. Aluminium, or other metallic salts, may be present as stabilizers.

* Dissolve 10–15 mg. purified 4-methyl-6-chlorothioindoxyl in 2–5 ml. acetone and add to 35 ml. 0·2 M-Tris buffer at pH 8·5. Filter before use. Use only freshly made solutions.

The majority of stable diazotates are produced by this last method and they contain some 5–6 per cent. of zinc chloride and often some aluminium salt in addition. A number of stable diazotates are available commercially and a list of these is given in the Table below. Additions are constantly being made as new demands occur, so that the list is inevitably incomplete.

Some Common Stable Diazotates

(see also Appendix 14, p. 870)

Seller*	Trade name	Diazotate of	Country †
Ciba . . .	Fast Red Salt V	5-nitroanisidine	Sw.
G. T. Gurr (I.C.I.) –	Fast Red Salt B	5-nitroanisidine	U.K.
I.G. Farb . .	Kernechtrotsalz B	5-nitroanisidine	G.
Bayer . . .	Azorotsalz B	5-nitroanisidine	G.
DuPont. . .	Naphthanil Diazo Red B	5-nitroanisidine	U.S.
G.D. Corp. . .	Diazo Fast Blue RR	4-benzoylamino-2 : 5-dimethoxyaniline	U.S.
Dajac (DuPont) .	Naphthanil Diazo Blue B	*o*-dianisidine	U.S.
G. T. Gurr (I.C.I.) .	Fast Blue B Salt	*o*-dianisidine	U.K.
G.D. Corp. . .	Fast Blue Salt BN	*o*-dianisidine	U.S.
Dajac (DuPont) .	Naphthanil Diazo Red RC	4-chloro-2-anisidine	U.S.
Dajac . . .	(None)	α-naphthylamine	U.S.
G. T. Gurr (I.C.I.) .	Fast Black Salt K	*p*-nitrobenzene-azo-dimethylaniline	U.K.
G. T. Gurr (I.C.I.) .	Fast Garnet	*o*-aminoazotoluene	U.K.
G.D. Corp. . .	Diazo Garnet GBC Salt	*o*-aminoazotoluene	U.S.

* Ciba — Ciba, Basel.
Bayer — Bayer, Leverkusen.
Dajac — Dajac Laboratories.
DuPont — DuPont de Nemours.
G.D. Corp — General Dyestuff Corporation.
G. T. Gurr — George T. Gurr Ltd., 136 King's Road, London, S.W.6.
I.C.I. — Imperial Chemical Industries Ltd., Manchester 9, England.
I.G. Farb. — I.G. Farbenindustrie.

† G. Germany. U.K. United Kingdom
Sw. Switzerland. U.S. United States of America.

GIBBS' METHOD FOR ARGENTAFFIN CELL GRANULES
(after Gomori)

(Formalin; paraffin sections. Avoid mercury)

Preparation of Staining Solution

Dissolve 20 mg. 2 : 6-dichloroquinone-chloroimide (Gibbs' reagent) in 20 ml. veronal acetate buffer at pH 9·2 by warming to 70° and shaking. Cool before use and filter.

Method

(1) Bring paraffin sections to water.
(2) Treat with the above solution for 10–15 minutes.
(3) Wash well in running water.
(4) Counterstain nuclei with 1 per cent. aqueous neutral red, 3 minutes.
(5) Dehydrate and differentiate the nuclear stain by rapid passage through 70 per cent. and absolute alcohol to xylene.
(6) Mount in DPX.

Result (Fig. 204)

Argentaffin granules, black. Nuclei, red. (Granules in carcinoid tumours are often brownish.)

THE SCHMORL METHOD FOR LIPOFUSCIN

(Formalin; frozen or paraffin sections)

Preparation of Ferricyanide Solution

Mix three parts of 1 per cent. ferric chloride or ferric sulphate and one part of freshly prepared 1 per cent. potassium ferricyanide. Use within 30 minutes.

Method

(1) Bring sections to water.
(2) Immerse in ferricyanide solution 5 minutes.
(3) Wash in running water.

For strictly histochemical investigations the section should be examined at this stage in water.

(4) Counterstain nuclei in 1 per cent. neutral red, 3 minutes.
(5) Dehydrate rapidly in alcohol, clear in xylene and mount in Canada balsam, or in a suitable synthetic resin.

Result (Fig. 205 and Plate VIIIᴅ)

Substances which reduce ferricyanide to ferrocyanide appear dark blue. These include melanin, argentaffin granules and lipofuscin as well as tissue components containing active sulphydryl groups.

METHOD FOR DISTINGUISHING MELANINS FROM LIPOFUSCINS (Hueck, 1912)

(Various fixatives; Paraffin sections)

Method

(1) Bring sections to water.
(2) Stain in a freshly-prepared saturated aqueous solution of Nile Blue sulphate, 30 minutes.
(3) Rinse in distilled water.
(4) Transfer to 3 per cent. (10 per cent. is better) H_2O_2 for 24 hours.
(5) Wash in running water.
(6) Mount in glycerine jelly or corn syrup.

Result

Lipofuscins, blue. Melanins, colourless.

ALTERNATIVE NILE BLUE METHOD (Lillie, 1956)

(Various fixatives; paraffin sections)

Method

(1) Bring paraffin sections to water.
(2) Stain for 20 minutes in 0·05 per cent. Nile blue A in 1 per cent. sulphuric acid.
(3) Wash in running water for 10–20 minutes.

(4) Alternatively rinse quickly in 1 per cent. sulphuric acid and dehydrate in 4 changes of absolute acetone.

(5) Mount from stage 3 into glycerine jelly and from stage 4, through xylene, in a synthetic resin.

Result

If stage 3–5 is employed lipofuscins stain dark blue or green, melanins dark green.

If stage 4–5 is employed melanins stain dark green and lipofuscins appear in their original colours.

THE CHROME ALUM HÆMATOXYLIN METHOD FOR LIPOFUSCINS (after Gomori, 1941)

(*Formalin, etc.; paraffin sections*)

Preparation of the Staining Solution

Mix equal parts of a fresh 1 per cent. aqueous hæmatoxylin and 3 per cent. chrome alum. Add to each 100 ml. of the mixture 2 ml. 5 per cent. potassium dichromate and 1 ml. 5 per cent. sulphuric acid. Allow the mixture to ripen for 2—3 days before use. (The solution retains its staining properties for 4–8 weeks.)

Method

(1) Bring sections to water.

(2) Oxidize sections for 1–2 minutes in acid permanganate solution (47·5 ml. 0·5 per cent. $KMnO_4$ and 2·5 ml. 3 per cent. sulphuric acid).

(3) Bleach for 1 minute in 1 per cent. oxalic acid and wash in water.

(4) Stain in chrome alum hæmatoxylin for 10 minutes.

(5) Differentiate in 1 per cent. acid alcohol.

(6) Counterstain in 1 per cent. aqueous eosin, 5 minutes.

(7) Wash in running water.

(8) Dehydrate rapidly in alcohol, clear in xylene and mount in DPX.

Result

Lipofuscins, blue-black. Nuclei, purple. Cytoplasmic structures, pink or red.

LONG ZIEHL-NEELSEN METHOD FOR ACID FAST LIPOFUSCINS

(*Formalin, etc.: paraffin sections*)

Method

(1) Bring sections to water.

(2) Stain in carbol fuchsin solution for 3 hours at 60°.

Basic fuchsin	10 g.
Phenol	50 g.
Alcohol	100 m.
Distilled water	1,000 ml.

(3) Wash in running water.

(4) Differentiate in 1 per cent. acid alcohol until the red cells are just faint pink.

(5) Counterstain lightly with Mayer's hæmalum or 0·5 per cent. toluidine blue.

(6) Wash in running water.
(7) Dehydrate in alcohol, clear in xylene, mount in DPX.

Result

Acid fast lipofuscins, bright red. Lipoproteins, pink. Nuclei, dark blue or blue.

If counterstaining is too strong the basophilia of the lipofuscin will lead to development of a purple colour in positive lipofuscins. If a cytoplasmic counterstain is desired, the best is Lendrum's (1947) saturated tartrazine in cellosolve.

DAM METHOD FOR FAT PEROXIDES (Glavind *et al.*, 1949)

(*Fresh frozen, formalin-fixed frozen sections*)

Preparation of Solutions

Solution A. 40 mg. hæmin.
10 ml. pyridine.
20 ml. glacial acetic acid.
(This solution is stable.)

Solution B. Just before use, dissolve 25 mg. leuco-3 : 5-dichloro-4 : 4′-dihydroxyphenylamine in 3·5 ml. absolute alcohol and add 5 ml. distilled water.

(The leuco compound is prepared by reducing 2 : 6-dichlorophenol-indophenol with the equivalent of ascorbic acid in 50 per cent. alcohol. The sodium salt is precipitated with NaCl and purified by repeated solution in alcohol and precipitation by the addition of water.)

Method

(1) Mount fresh frozen sections on slides.
(2) Fix, if desired, for 5 minutes in 40 per cent. formalin, 1 part, 40 per cent. alcohol, 3 parts.
(3) Wash in water, remove and blot dry.
(4) Mix 0·74 ml. Solution A and 8·5 ml. Solution B and apply for 3–5 minutes.
(5) Wash in distilled water.
(6) Mount in glycerine jelly.

Result

Fat peroxides appear deep red.

DIAMINOFLUORENE METHOD FOR FAT PEROXIDES (Feigl, 1954)

(*Fresh sections*)

Method

(1) Mount 15μ fresh frozen sections on slides or coverslips.
(2) Cover section with 2 or 3 drops of the reagent* and apply coverslip.
(3) Examine under the microscope.

* Dissolve 100 mg. 2, 7-diaminofluorene and 5 mg. haemin in 5 ml. acetic acid. This reagent must be freshly prepared.

Result

A violet, blue or greenish-blue colour constitutes a positive reaction.

DIPHENYLCARBAZIDE REACTION FOR HYDROXY FATTY ACIDS (Feigl, 1954)

(*Fresh sections*)

Method

(1) Treat fresh frozen sections with 0·5 per cent. diphenylcarbazide in 50 : 50 alcohol-ether.
(2) Rinse in alcohol.
(3) Bring to water and mount in glycerine jelly.

Result

Hydroxy fatty acids give a red colour.

PHLOROGLUCINOL METHOD FOR EPIHYDRINALDEHYDES (Feigl, 1954)

(*Fresh sections*)

Method

(1) Mount fresh sections on slides or coverslips. Store in air for 7–28 days.
(2) Immerse in 0·1 per cent. phloroglucinol in 96 per cent. alcohol containing 10 per cent. HCl. Incubate at 40° for 15 minutes.
(3) Rinse in alcohol and bring to water.
(4) Mount in glycerine jelly.

Result

Epihydrinaldehydes give a deep red colour.

BENZIDINE METHOD FOR HÆMOGLOBIN (after Pickworth, 1934–35)

(*Formalin etc.; paraffin sections*)

Preparation of Solutions

Benzidine. Dissolve 0·2 g. benzidine and a small (5 mm.^{3}) crystal of sodium nitroprusside in 15 ml. methanol. Add 4 drops of glacial acetic acid and shake.

Ozonic Ether.

3 per cent. H_2O_2 . . .	50 ml.
Methanol	100 ml.
Ether	50 ml.

Method

(1) Bring sections to water.
(2) Rinse in methyl alcohol.
(3) Invert the slide over benzidine solution on a staining tile. Leave for 5–10 minutes.
(4) Wash off benzidine with ozonic ether.
(5) Leave slide for 10 minutes in fresh ozonic ether.
(6) Wash in running water 10–15 minutes.

(7) Counterstain nuclei in 1 per cent. aqueous neutral red, 3 minutes.
(8) Wash, dehydrate in alcohol, clear in xylene and mount in DPX.

Result

Hæmoglobin and certain "oxidase" granules in leucocytes, dark blue. Nuclei, red.

THE GMELIN REACTION FOR BILIRUBIN AND HÆMATOIDIN

(*Formalin, alcohol; paraffin sections*)

Method

(1) Bring sections to water.
(2) Apply a few drops of a mixture of equal parts of conc. nitric acid and absolute alcohol.
(3) Apply a coverslip and wipe off excess reagent with blotting-paper.
(4) Ring with paraffin wax to seal edges (optional).
(5) Examine under microscope for development of colour.

Result

A spectrum of colours from red through purple to green is observed to spread outwards from masses of bilirubin and from hæmatoidin crystals.

THE IODINE METHOD FOR BILE PIGMENTS (Stein, 1935)

(*Formalin, alcohol; paraffin sections*)

Solutions

(1) **Iodine Reagent.** Lugol's iodine . . 2 parts
Tinct. iod. . . . 1 part
(2) **Five per cent. Sodium Thiosulphate.**

Method

(1) Bring sections to water.
(2) Treat with the iodine reagent for 12–18 hours.
(3) Wash in running water for 5 minutes.
(4) Decolorize with thiosulphate solution, 30 seconds.
(5) Wash and counterstain with Mayer's carmalum, 3–18 hours.
(6) Wash in water, dehydrate in absolute acetone, clear in xylene, mount in balsam.

Result

Bile pigments appear dark green. Nuclei, red.

FERRIC IRON METHODS FOR BILIRUBIN (Kutlik, 1957)

(*Formalin, Carnoy etc.; paraffin sections*)

Method

(1) Bring paraffin sections to water.
(2) Immerse for 15 minutes in 5 per cent. aqueous iron alum or for 10 minutes in 5 per cent. $FeCl_3$ in 2 per cent. acetic acid.
(3) Wash briefly in distilled water.
(4) Rinse in 96 per cent. alcohol.

(5) Counterstain with 1 per cent. Brilliant Yellow (Geigy) in 96 per cent. alcohol, 10 minutes.

(6) Rinse in 96 per cent. alcohol.

(7) Blot dry, transfer to xylene and mount in Canada balsam.

Result

Bilirubin is converted into green biliverdin. Background yellow.

TRICHROME METHOD FOR BILIRUBIN, HÆMOSIDERIN AND LIPOFUSCIN (Glenner, 1957)

(*Fresh sections*)

Method

(1) Cut thin (10μ) cold microtome sections and mount on slides.

(2) Immerse for 5 minutes in 2 per cent. potassium ferricyanide.

(3) Place in acetic-ferricyanide for 20 minutes. (Freshly mixed, equal parts of 2 per cent. ferricyanide and 5 per cent. acetic acid.)

(4) Rinse in running water and immerse in 3 per cent. potassium dichromate at pH 2·2 for 15 minutes.

(5) Wash in water.

(6) Place sections in freshly filtered Oil red O in *iso*propanol (see p. 854). Stain for 20 minutes.

(7) Wash in running water for 5 minutes.

(8) Mount in Apáthy's syrup.

Result

Lipofuscins dark orange red, hæmosiderin blue, bilirubin dark emerald green.

APPENDIX 24

PERLS' METHOD FOR FERRIC IRON (after Lison and Bunting)

(Formalin-fixed, paraffin sections preferable)

Acid fixatives must be avoided, chromates also interfere with the preservation of iron.

Method

(1) Bring sections to distilled water.
(2) Expose sections to a fresh mixture of equal parts of 2 per cent. potassium or sodium ferrocyanide and 2 per cent. HCl for 30–60 minutes. (If the longer period is to be used, change the solution after 30 minutes.)
(3) Wash in distilled water.
(4) Counterstain nuclei with 1 per cent. aqueous neutral red, 3 minutes.
(5) Wash, dehydrate in alcohol, clear in xylene and mount in DPX.

Result (Fig. 209)

Ferric iron, deep Prussian blue. Nuclei, red.

TURNBULL BLUE METHOD FOR FERRIC IRON (after Tirman and Schmeltzer)

(Formalin-fixed, paraffin sections)

Method

(1) Bring sections to distilled water.
(2) Treat the section for 1–3 hours with a dilute solution of yellow ammonium sulphide.
(3) Rinse in distilled water.
(4) Treat with a freshly-prepared mixture of equal parts of 20 per cent. potassium ferricyanide and 1 per cent. HCl for 10–20 minutes.
(5) Rinse in distilled water.
(6) Counterstain nuclei red, if desired.
(7) Dehydrate, clear and mount.

Result

Ferrous iron, and ferric iron converted to this state, deep blue.

THE DINITROSORESORCINOL METHOD FOR FERRIC IRON (after Humphrey, 1935)

(Formalin-fixed, paraffin sections)

Method

(1) Bring sections to distilled water.
(2) Treat with 10 per cent. yellow ammonium sulphide for 1 hour.
(3) Rinse in distilled water.
(4) Treat with a 3 per cent. solution of dinitroresorcinol in 50 per cent. alcohol for 6–18 hours.
(5) Wash in 50 per cent. alcohol.
(6) Dehydrate in absolute alcohol, clear in xylene, mount in DPX.

Result

Ferrous iron, and ferric iron converted by treatment with sulphide, appear greenish-blue. Background structures brown.

This method is insensitive by comparison with Perls' technique and the deep colour of the background obscures some of the finer deposits. Diffuse iron is not demonstrated by this method, which is only occasionally useful when avoidance of iron containing reagents is thought necessary.

HÆMATOXYLIN-LAKE METHOD FOR HÆMOSIDERIN IRON (after Macallum, Mallory and Bunting)

Method

(1) Proceed through stages 1–3 of the dinitroresorcinol method.

(2) Stain for 2–6 hours in a freshly-prepared 0·5 per cent. hæmatoxylin in 30 per cent. ethanol. (Prepare by dissolving 10 mg. hæmatoxylin in 2 ml. absolute ethanol and adding 4 ml. distilled water.)

(3) Wash in absolute alcohol, then in 50 per cent. alcohol and in distilled water.

(4) Counterstain (optional) in Mayer's carmalum 1–6 hours.

(5) Wash in water, dehydrate, clear and mount in DPX.

Result

Hæmosiderin appears blue-black. Nuclei, red.

CADMIUM SULPHATE METHOD FOR FERRITIN AND APOFERRITIN (Granick, 1946)

(Fresh frozen, cold knife or cold microtome, teased)

Method

(1) Teased portions of the fresh tissues, weighing about 0·2 g., or fresh frozen sections should be kept overnight at room temperature in 10 per cent. aqueous cadmium sulphate. This may be done on the slide, under a ringed coverslip, or in a small amount of the cadmium solution in a closed vial.

Result

If ferritin was present the typical yellow octahedral crystals of the cadmium-ferritin complex will be visible under the microscope.

ALIZARIN RED S METHOD FOR CALCIUM (Dahl, 1952)

(Alcohol-fixed, paraffin sections)

Mount sections without adhesive.

Preparation of Solutions

(1) *Alizarin Red S.* Stir 0·5 g. of the dye in 45 ml. distilled water and add 5 ml. of a 1 : 100 dilution of 28 per cent. NH_4OH, with constant stirring. The pH should be between 6·3 and 6·5 and the solution keeps for at least a month.

(2) *Differentiation.* 10^{-3} M-HCl in 95 per cent. ethanol (conc. HCl 1 part ; 95 per cent. ethanol 10,000 parts).

Method

(1) Bring sections to water.
(2) Stain with Alizarin solution for 2 minutes.
(3) Wash with distilled water for 5–10 seconds.
(4) Rinse in acid ethanol for 15 seconds.
(5) Dehydrate, clear in xylene and mount in cedarwood oil.

Result

Calcium deposits appear orange-red. Inorganic iron (not hæmosiderin), purple.

With the older alizarin techniques stress was laid on the avoidance of acids for differentiation because of the danger of removal of calcium. The acid wash employed here is of brief duration and it is improbable that any calcium is dissolved.

GYPSUM METHOD FOR CALCIUM

(Formalin-fixed, paraffin sections)

Method

(1) Bring sections to acetone and allow to dry in air.
(2) Place a drop of 5 per cent. sulphuric acid on the area of the section where the deposits are located.
(3) Observe under low power objective for the formation of the characteristic long thin needles of hydrated calcium sulphate ($CaSO_4.2H_2O$). The needles usually change into thin prisms and finally into masses of plates piled on top of one another.

CALCIUM RED METHOD FOR CALCIUM
(McGee-Russell, 1955)

(Formalin or alcohol, paraffin sections)

Preparation for Staining Solution

Wash 2 g. Nuclear Fast red (G. T. Gurr ; Batch 3569 recommended), twice, with about 100 ml. distilled water. Take up the residue (about 0·25 g.) with 100 ml. distilled water.

Method

(1) Bring sections to water.
(2) Stain in calcium red solution 1–10 minutes.
(3) Wash in distilled water.
(4) Dehydrate in alcohols, clear in xylene and mount (synthetic media).

Result

Calcium deposits, red. Tissues, various shades of pink.

PHTHALOCYANIN METHOD FOR CALCIUM

(Formalin or alcohol, paraffin sections)

Method

(1) Bring two sections to water.
(2) Treat one section with 1 per cent. aqueous HCl for 20 minutes, wash briefly in water.

(3) Stain for 30–60 minutes in a 0·1 per cent. aqueous solution of Durazol Fast blue S G with or without 0·1 per cent. Alcian blue 8 GS (I.C.I. Ltd.), or of any suitable sulphonated copper phthalocyanin dyes.

(4) Wash in running water for 1 minute.

(5) Differentiate in 0·05 per cent. lithium carbonate until the tissues in general are pale blue.

(6) Dehydrate, clear and mount in a suitable synthetic medium.

Result (Fig. 213)

Calcium deposits appear bright blue in the untreated section only; tissues stain blue in various degrees, unaffected by acid treatment.

Crystalline, birefringent, calcium phosphate deposits, and some amorphous ones, may stain more readily with dilute solutions of spirit-soluble copper phthalocyanins in 85–95 per cent. ethanol. Methanol Fast blue 2 G (I.C.I. Ltd.) and Luxol Fast blue MBS (Dupont) are examples of dyes of this type.

ERIOCHROME BLACK T METHOD FOR CALCIUM
(Invertebrates only)

(*Formalin, etc.; paraffin sections*)

Method

(1) Bring paraffin sections to water.

(2) Stain for 3–5 minutes in 0·5 per cent. aqueous Eriochrome black T buffered to pH 9·5 with N-NaOH.

(3) Wash in distilled water.

(4) Mount in glycerine jelly.

Result

Calcium deposits stain bright red. Tissues generally stain in various shades of blue.

THE VON KÓSSA METHOD FOR "CALCIUM" DEPOSITS

(*Formalin-fixed, paraffin sections, etc.*)

Method

(1) Bring sections to distilled water and rinse thoroughly.

(2) Immerse in 0·5–1 per cent. aqueous $AgNO_3$ for 10–15 minutes. (This procedure should be carried out in sunlight or ultraviolet light.)

(3) Rinse in distilled water.

(4) Immerse in 5 per cent. aqueous sodium thiosulphate, 30 seconds.

(5) Wash in tap water.

(6) Counterstain nuclei with 1 per cent. neutral red, 30 seconds.

(7) Dehydrate in alcohol, clear in xylene and mount in Canada balsam or DPX. (The latter causes slow fading.)

Result

Phosphates and carbonates appear black. Nuclei, red.

SOLOCHROME AZURINE METHOD FOR ALUMINIUM

(*Formalin-fixed paraffin, etc.*)

Method

(1) Bring sections to water.
(2) Stain for 15–20 minutes in 0·2 per cent. aqueous Solochrome azurine (I.C.I. Ltd.).
(3) Rinse in distilled water.
(4) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic medium.

Result (Plate IXB)

Aluminium deposits appear deep steel blue ; beryllium stains similarly. If distinction is required this can be made by means of alkali pretreatment which removes aluminium hydroxide.

FLUORESCENT (MORIN) METHOD FOR CALCIUM AND ALUMINIUM

(*Cold knife, cold microtome; formalin-fixed frozen sections, formalin-fixed paraffin sections*)

Cold-knife or cold-microtome sections may be delivered straight into the staining solution. Other sections are brought to 85 per cent. ethanol. A control section pretreated with 1 per cent. aqueous HCl for 10 minutes should always be included.

Method

(1) Stain for 2–5 minutes in 0·2 per cent. morin in 85 per cent. ethanol containing 0·5 per cent. acetic acid.
(2) Wash briefly with 70 per cent. ethanol, or, if overstained, with 1 per cent. acid alcohol.
(3) Wash in distilled water.
(4) Mount in water and ring coverslip with paraffin wax. If a more permanent mount is required the section should rapidly be dehydrated in the alcohols, cleared in xylene and mounted in fluormount (E. Gurr Ltd.).

Result (Figs. 216, 217 and 219)

In the section untreated with HCl a bright greenish-white fluorescence, absent from the control section, indicates the presence of calcium. Aluminium hydroxide, which is acid-stable, gives a more green fluorescence than calcium. On occasion the edges of the section may show an even fluorescence of greenish colour regardless of structure. In such cases only the inner part of the section where the background fluorescence varies from dull to bright yellow, should be used for critical assessments.

AURINE METHODS FOR ALUMINIUM HYDROXIDE (Irwin, 1955)

(*Formalin-fixed paraffin sections, etc.*)

Preparation of Solutions

(1) *Aurine buffer Solution.* Mix 60 ml. 5 M-ammonium chloride, 60 ml. 5 M-ammonium acetate, 10 ml. 6 N-HCl.

This should give ± pH 5·2.

Dissolve 2 g. aurine tricarboxylic acid (Aluminon) in a few ml. of the buffer and make up to 100 ml. Heat to 75° for 15 minutes and filter while hot.

(2) *Differentiating Solution.* Mix 50 ml. of the buffer solution (above), 22 ml. of 1·6 M-ammonium carbonate.

The pH of this solution should be about 7·2.

Method

(1) Bring sections to water.
(2) Stain for 5–10 minutes in buffered aurine at 75°.*
(3) Rinse in distilled water.
(4) Differentiate in ammonium carbonate solution for 3–5 seconds.
(5) Wash in distilled water.
(6) Counterstain in 1 per cent. aqueous methylene blue.
(7) Rinse in distilled water.
(8) Dehydrate in alcohols, clear in xylene and mount in a suitable permanent medium.

Result (Fig. 218)

Aluminium hydroxides appear as the bright red aurine lake. Nuclei, blue.

FLUORESCENT (SOLOCHROME) METHOD FOR ALUMINIUM SALTS

(*Formalin, frozen or paraffin sections, etc.*)

Method

(1) Bring paraffin sections to water.
(2) Treat with a saturated solution of Solochrome black B (I.C.I. Ltd.) in absolute ethanol containing 2 per cent. HCl, for 20–60 minutes.
(3) Rinse in absolute ethanol (paraffin sections) or in 1 per cent. acid alcohol (frozen sections).
(4) Bring frozen sections to water and mount in glycerine. Clear paraffin sections in xylene and mount in fluormount.

Result (Plate IXA)

Aluminium salts fluoresce in colours which vary from yellowish-orange to bright brick-red on a light blue background.

Calcium salts are first dissolved and then reprecipitated diffusely as the red Solochrome lake in approximately their original location. These Calcium-Solochrome lakes do not fluoresce.

ACID SOLOCHROME CYANINE METHOD FOR ALUMINIUM

(*Formalin-fixed paraffin, etc.*)

Method

(1) Bring sections to water.
(2) Stain in 0·2 per cent. Solochrome cyanine RS (I.C.I. Ltd.) in 1 per cent. aqueous HCl for 15–30 minutes.

* At this temperature there is a tendency for the sections to float off the slides. Longer incubation at 60° may be tried.

(3) Rinse in running hot tap water until the section has changed from yellow, through red to a pale grey-blue colour.

(4) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic medium.

Result (Plate IXc)

Aluminium deposits appear deep rose red to pink, depending on the amount of aluminium present. Nuclei, bright steel blue. Red blood cells, bright red. Basic proteins, pinkish-red or red.

Differentiation in 1 per cent. acid alcohol before this stage removes the nuclear and red cell counterstain, leaving only aluminium deposits stained.

NAPHTHOCHROME GREEN B METHOD FOR ALUMINIUM, CALCIUM AND BERYLLIUM (after Denz, 1949)

(*Formalin-fixed frozen sections, formalin- or alcohol-fixed paraffin sections*)

Method

(1) Bring sections to water.

(2) Stain in 0·1 per cent. Naphthochrome green B in 0·1 M-phosphate buffer (pH 5·0) for 30 minutes at 37° (beryllium).

(2A) Stain in 0·1–0·2 per cent. aqueous Naphthochrome green B at room temperature for 5–10 minutes (calcium, aluminium).

(3) Wash in distilled water.

(4) Differentiate for up to 30 minutes (if necessary) in absolute alcohol (Beryllium).

(4A) Dehydrate, clear and mount in synthetic medium (calcium, aluminium).

(5) Wash in distilled water (beryllium).

(6) Counterstain if desired (Denz recommended 1 per cent. acridine red).

(7) Dehydrate, clear, and mount, etc.

Result (Figs. 215 and 220)

Beryllium (hydroxide) or beryllium salt/protein complexes stain apple green.

Calcium salts appear brown to brownish-red and aluminium hydroxide a rich deep green. (If other metals (Fe^{+++}) are present calcium deposits stain green. After treatment with mercuric salts most calcium deposits appear green but in the case of bony trabeculæ the older parts stain red and the newer parts green. Osteoid does not stain.

A method for the purification of this dye is given by Aldridge and Liddell (1948). I have not found purification of presently available dye samples at all necessary.

COPPER METHOD FOR ARSENITES AND ARSENATES (Castel, 1936)

(*Formalin-fixed paraffin sections*)

Method

(1) Fix tissues (thin blocks) in 10 per cent. formalin containing 2·5 per cent. $CuSO_4 5H_2O$ for 5 days.

(2) Wash in running water for 24 hours.

(3) Dehydrate, clear and embed in paraffin wax.

(4) Cut sections (preferably thick ones), remove wax and mount without counterstaining.

Result

A green granular deposit indicates the presence of salts of arsenic.

ALKALINE SOLOCHROME AZURINE METHOD FOR BERYLLIUM

(*Formalin-fixed paraffin sections, etc.*)

Method

(1) Bring sections to water.

(2) Stain for 30 minutes in a 0·2 per cent. solution of Solochrome azurine (I.C.I. Ltd.) in 2 per cent. NaOH.

(3) Rinse in distilled water.

(4) Dehydrate in alcohols, clear in xylene and mount in a suitable synthetic medium.

Result (Plate IXD)

Beryllium deposits appear almost black ; treatment with acid converts them to a brownish-orange. Aluminium deposits ($AlOH_2$) are dissolved in the staining baths, calcium deposits by treatment with dilute acids.

ALKALINE QUINALIZARIN METHOD FOR BERYLLIUM AND CALCIUM

(*Formalin-fixed frozen sections, formalin- or alcohol-fixed paraffin sections, etc.*)

Method

(1) Bring sections to water.

(2) Stain in 0·2 per cent. quinalizarin (1,2,5,8-tetrahydroxy anthraquinone) in 4 per cent. NaOH for 3–5 minutes.

(3) Wash carefully in distilled water.

(4) Mount frozen sections in glycerine jelly, preferably without counterstain.

(4A) Dehydrate paraffin sections in alcohols, clear in xylene and mount in a suitable synthetic medium.

Result (Fig. 221)

Beryllium and calcium deposits stain deep purple. Nuclei may begin to stain, especially if time of staining is prolonged or if the concentration of NaOH is reduced.

RHODIZONATE METHOD FOR BARIUM SALTS

(*Formalin, frozen or paraffin sections, etc.*)

Method

(1) Bring sections to water.

(2) Transfer to a freshly-prepared 0·2 per cent. aqueous solution of sodium rhodizonate. Incubate at 37° (frozen sections) or at 60° (paraffin sections) for 1–2 hours.

(3) Wash briefly in water.

(4) If desired convert the black rhodizonate to the red acid salt by immersion in 20 per cent. HCl for a few minutes.

(5) Wash again in water.
(6) Dehydrate, clear and mount in a synthetic medium.

Result

Barium deposits black, converted to scarlet-red by acid treatment. Strontium salts, which react similarly, would be dissolved by this treatment.

BRUCINE-IODIDE METHOD FOR BISMUTH (after Wachstein and Zak)

(Formalin-fixed, frozen or paraffin sections)

Preparation of Solutions

(1) **Modified Castel Reagent.** Dissolve 0·25 g. brucine sulphate in 100 ml. distilled water with 3 drops conc. sulphuric acid. Stir until the salt dissolves and add 2 g. potassium iodide. Store in a brown bottle and filter before use.
(2) **Dilute Castel Reagent.** Dilute the stock reagent 1 : 3 with distilled water.
(3) **Thirty per cent. (100 vol.) H_2O_2.**
(4) **Lævulose Solution.** Dissolve 30 g. lævulose in 20 ml. water by warming at 37° for 24 hours and add a drop of the dilute Castel reagent.
(5) **Counterstain.** Add 1 ml. 1 per cent. aqueous light green SF to 100 ml. undiluted Castel reagent. Filter before use.

Method

(1) Bring sections to water and blot dry.
(2) Apply 30 per cent. H_2O_2 for 10–15 seconds.
(3) Wash in running water.
(4) Place in modified Castel reagent for 1 hour.
(5) Transfer to dilute Castel reagent and shake gently to remove precipitates.
(6) Counterstain for 4 minutes and wash in water.
(7) Mount in lævulose solution.

Result

Bismuth appears as an orange-red deposit whose colour may darken on standing.

DIMETHYLGLYOXIME METHOD FOR NICKEL SALTS (Feigl, 1954)

This method has been applied by Wells (1956) to formalin-fixed or fresh frozen skin sections treated for short periods with 1 per cent. aqueous nickel sulphate.

Method

(1) Treat sections with a saturated solution of dimethylglyoxime in alcohol at 37° for 10–20 minutes.
(2) Rinse in absolute alcohol.
(3) Clear in xylene and mount in a suitable synthetic medium.

Result

Sites of nickel adsorption appear in shades of pink.

RUBEANIC ACID METHOD FOR COPPER (Okamoto, 1938 ; Uzman, 1956)

(*Cold knife and cold microtome, fresh slices, formalin-fixed, frozen or paraffin sections, etc.*)

The method as described is for fresh slices 1–2 mm. thick. It may easily be adapted for application to fresh frozen or formalin-fixed frozen sections, or to paraffin sections. The latter may first be treated as described below in order to make bound copper available for reaction.

Method

(1) Immerse thin slices of fresh tissues in 0·1 per cent. rubeanic acid in 70 per cent. ethanol for 10 minutes.

(2) Add sodium acetate (analytical reagent grade) at 200 mg./100 ml. to the above solution and leave for 24–48 hours.

(3) Wash with 70 per cent. ethanol and stand in this for 60–90 minutes.

(4) Replace with absolute ethanol and stand for 24 hours.

(5) Clear in chloroform and embed in paraffin wax.

(6) Cut sections and mount on slides (egg-albuminized).

(7) Remove wax with xylene and mount in Canada balsam.

Result (Fig. 222)

Copper salts or protein complexes appear as black precipitates.

Application to Paraffin Sections

In order to release protein-bound copper the sections are dewaxed with xylene and then placed face downwards over a beaker of conc. HCl for 15 minutes. They are then treated with absolute ethanol for 15 minutes and then transferred to the rubeanic acid bath.

DIETHYLDITHIOCARBAMATE METHOD FOR COPPER (after Waterhouse, 1945)

(*Cold microtome, cold knife, teased, or freshly-dissected whole specimens*)

Method

(1) Stain in aqueous 0·1–0·2 per cent. sodium diethyldithiocarbamate, with or without 2 per cent. HCl, for 1–3 hours.

(2) Wash in distilled water and fix in 10 per cent. formalin. (In the case of fresh frozen sections 1 ml. of 40 per cent. analytical reagent formaldehyde may be added to 10 ml. of the staining medium after staining is complete. The tissues are allowed to fix before washing and mounting.)

(3) Transfer to clean slides and mount in glycerine jelly.

Result (Fig. 224)

Copper salts appear yellowish-brown.

O-TOLIDINE-THIOCYANATE METHOD FOR COPPER

(*Cold knife, cold microtome, formalin- or formol-calcium-fixed frozen sections, formol-calcium-fixed paraffin sections*)

Method

(1) Bring paraffin sections to water.

(2) Flood the section with a mixture containing acetone, 5 ml. ; water,

5 ml. ; *o*-tolidine, 50 mg. ; ammonium thiocyanate, 500 mg. Frozen sections should be immersed in a small dish of this medium, and they should be mounted on slides and blotted dry before the next stage.

(3) Rinse in 70 per cent. alcohol.

(4) Dehydrate in absolute alcohol and clear in xylene.

(5) Mount in Canada Balsam or Gurr's Neutral Mountant (G. Gurr Ltd., London).

Result

Copper salts are indicated by the presence of a bright blue colour, diffuse or more often crystalline. This is unstable and it disappears rapidly in mounting media which contain oxidizing or reducing agents.

BENZIDINE METHOD FOR COPPER

(*Formalin, paraffin sections*)

Method

(1) Bring sections to water.

(2) Cover with a solution containing 10 mg. benzidine hydrochloride and 30 mg. ammonium thiocyanate in 5 ml. distilled water. Leave for 5–10 minutes.

(3) Rinse in distilled water.

(4) Counterstain in 1 per cent. aqueous neutral red, 15 seconds.

(5) Rinse in water.

(6) Dehydrate in the alcohols, clear in xylene and mount in Canada balsam or Gurr's neutral mounting medium.

Result

Copper, if present as Cu^{++}, shows as an amorphous or sometimes crystalline deposit of benzidine blue.

This method has not been applied successfully to mammalian tissues except when copper salts have been introduced artificially.

REDUCTION METHOD FOR INORGANIC GOLD SALTS (Christeller, 1927)

(*Formalin- or alcohol-fixed paraffin sections*)

Method

(1) Bring sections to water.

(2) Incubate for 36 hours at 56° in a solution containing 40 ml. freshly-prepared 5 per cent. $SnCl_2$ and 5 ml. of concentrated HCl.

(3) Wash carefully in water.

(4) Counterstain the background if desired (carmalum).

(5) Dehydrate, clear and mount in the usual way.

Result

Gold appears in the form of black or brownish granules.

SULPHIDE METHOD FOR LEAD SALTS (after Timm, 1936)

This method is especially useful for the demonstration of lead deposits in bone.

Method

(1) Fix thin pieces of tissue in 10 per cent. formol saline saturated with H_2S, or in absolute ethanol similarly saturated, for 48–72 hours.

(2) Wash in running water for 6 hours.

(3) Decalcify if necessary in 10–30 per cent. formic acid, saturated with H_2S.

(4) Remove acid by treatment with several changes of 5 per cent. sodium sulphate and then with water, both being saturated with H_2S.

(5) Cut frozen sections at 10–20 μ.

(6) Transfer sections to slides and mount in glycerine jelly.

Result

Lead appears as brownish granules of PbS.

CHROMATE METHOD FOR LEAD SALTS (Cretin, 1929 ; modified by Fairhall, 1940)

(Formalin-fixed paraffin sections or fresh tissues)

Method

(1) Fix fresh tissues in Regaud's (80 ml. 3 per cent. potassium dichromate with 20 ml. 40 per cent. formaldehyde freshly added), 24–48 hours.

(1A) Treat paraffin sections, after bringing to water, with a 2 per cent. potassium chromate solution in 1 per cent. aqueous acetic acid. A time of several days is recommended.

(2) Wash tissues in running water for 6 hours.

(3) Dehydrate, clear and embed in paraffin wax.

(4) Cut paraffin sections at 5–10 μ and bring to water.

(5) Counterstain sections from either source with 0·5 per cent. toluidine blue (1 minute).

(6) Dehydrate, clear and mount in a suitable synthetic medium.

Result

Lead salts appear as yellow crystals of the chromate, on a pale blue background.

RHODIZONATE METHOD FOR LEAD SALTS

(Cold microtome or cold knife, fresh sections ; formalin-fixed paraffin sections)

Bones containing lead salts might well be decalcified in 5–10 per cent. H_2SO_4 containing 5–10 per cent. Na_2SO_4 (Gomori 1952). This procedure should transform Pb deposits into insoluble $PbSO_4$.

Method

(1) Transfer frozen sections (15–20 μ) directly into a freshly-prepared 0·2 per cent. solution of sodium rhodizonate in 1 per cent. acetic acid. Leave for 30–60 minutes.

(2) In the case of unfixed sections add 40 per cent. formaldehyde to the rhodizonate medium and leave to fix for 30–60 minutes.

(3) Transfer sections to slides and counterstain if desired with 0·1 per cent. light green S.F. in 1 per cent. acetic acid (30 seconds).

(4) Mount in glycerine jelly.

Result

Lead salts appear scarlet-red, background light green.

THE TITAN YELLOW METHOD FOR MAGNESIUM

(*Mounted fresh frozen sections, formalin-fixed frozen and paraffin sections, etc.*)

Method

(1) Cover the section with a few drops of 0·2 per cent. aqueous Titan yellow.
(2) Add a similar quantity of 2 N-NaOH and leave for 45–60 minutes.
(3) Wash off the reaction mixture.
(4) Dehydrate in alcohols.
(5) Clear in xylene and mount in DPX or other suitable synthetic medium.

Result (Fig. 225)

Magnesium salts appear in a rich flame-red colour which is unstable and fades in about 24 hours.

THE MAGNESON METHOD FOR MAGNESIUM

(*Fresh frozen, formalin-fixed frozen or paraffin sections, Carnoy, etc.*)

Method

(1) Incubate sections at 56°–60° for 30 minutes in a 1 per cent. solution of magneson II * in 5 per cent. NaOH (pH 12·2).
(2) Wash in dilute NaOH.
(3) Dehydrate in alcohols.
(4) Clear in xylene and mount in DPX or other suitable synthetic medium.

Result (Fig. 226)

Magnesium salts are coloured bright blue.

p-DIMETHYLAMINOBENZYLIDENE RHODANINE METHOD FOR SILVER (Okamoto *et al.*, 1939)

(*Cold knife or cold microtome sections, fresh slices, formalin- or alcohol-fixed paraffin sections*)

Preparation of Solutions

Rhodanine Solution. Dissolve 0·2 g. *p*-dimethylaminobenzylidene rhodanine in 100 ml. of 10 per cent. ethanol containing 0·1 per cent. nitric acid.

Method

(1) If possible, cut fresh tissue sections (20 μ) into the staining solution. Alternatively, fix and stain thin slices of tissue in it, in both cases for 1–3 hours at 37°. If no other is available paraffin sections may be treated for 24 hours at 37° in Okamoto's original mixture (saturated alcoholic solution of the reagent, 3·5 ml. ; N-HNO_3, 3 ml. ; water, 93·5 ml.).

* *p*-Nitrobenzene-azo-1-naphthol.

(2) Wash the free floating sections in 70 per cent. ethanol.
(3) Bring to water and mount on slides in glycerine jelly.

Result

Silver deposits appear reddish-violet.

METHOD OF GERSH FOR POTASSIUM

(*Freeze-dried material only*)

All operations, the first excepted, must be carried out in a cold room at 0°.

Method

(1) Cut paraffin sections at 15 μ and mount dry on coverslips by the application of finger pressure.
(2) Remove wax with light petroleum and dry section by burning off excess petroleum (condensation produced by evaporation is thus avoided).
(3) Cover the section with a drop of 12 per cent. aqueous sodium cobaltinitrite.
(4) Allow to dry and mount directly in glycerine.
(5) Examine microscopically at 0°.

Result

Yellowish crystals and microcrystals of potassium cobaltinitrite indicate the presence of potassium salts.

The sensitivity of this reaction is not high (Feigl, 1954, gives 4 γ potassium as the limit in spot tests), and unless there is a high concentration of potassium in a given situation conditions necessary for the formation of a precipitate will not be present. Under these circumstances caution is necessary in interpreting results.

MODIFIED GERSH METHOD FOR POTASSIUM (Crout and Jennings, 1957)

Method

(1) Cut freeze-dried sections at 10 μ and transfer directly into light petroleum at room temperature. Pick up sections and transfer to a fresh bath of light petroleum.
(2) Mount sections on a clean dry slide. Remove from light petroleum and allow to dry in the air. Press section firmly on to the slide.
(3) Immerse the slide in ice-cold cobaltinitrite * stain for 15 minutes.
(4) Withdraw the slide and blot off excess stain. Rinse in two changes of ice water until no more yellow material flows from the section (about 15 seconds).
(5) Dehydrate in absolute alcohol.
(6) Clear in xylene and mount in a suitable synthetic resin.

Result

Fine yellowish-brown granules indicate potassium, but the degree of accuracy of localization is uncertain.

* Dissolve 25 g. of cobaltous nitrite in 50 ml. distilled water ; add 12·5 ml. acetic acid. Dissolve 120 g. sodium nitrite in 180 ml. distilled water. Add to the solution of cobalt nitrate 210 ml. of the nitrite solution and draw air through the mixture until all the nitric oxide passes off (1–2 hours). Store in refrigerator and filter before use.

MOLYBDATE METHOD I FOR INORGANIC PHOSPHATES (after Serra and Feigl)

(*Formalin-fixed paraffin sections*)

Method

(1) Bring sections to water, and shake off excess.

Cover with a mixture of equal parts of 5 per cent. ammonium molybdate and 1 per cent. nitric acid, for 3–5 minutes, not longer.

(3) Wash in running water.

(4) Cover with benzidine solution (0·05 g. benzidine and 10 ml. of glacial acetic acid, made up to 100 ml. with distilled water). Leave for 1 minute.

(5) Pour off benzidine solution and flood section with 30 per cent. sodium acetate.

(6) Mount in sodium acetate and ring the coverslip with paraffin wax.

(Permanent sections cannot be obtained.)

Result (Fig. 211)

A blue colour indicates the presence of phosphate. Localization is not strictly accurate.

MOLYBDATE METHOD II FOR INORGANIC PHOSPHATES (Cheng, 1956)

(*Formalin-fixed frozen sections*)

Method

(1) Fix tissue slices 1 mm. thick in 50 per cent. formalin neutralized by passage through a column of activated ion-exchange resin (De-Acidite ; Permutit Co. Ltd.) before use.

(2) Cut frozen sections 20–50 μ thick and rinse these in acetate buffer at pH. 4·0 (0·1 N-acetic acid and 0·025 N-sodium acetate).

(3) Transfer to acid molybdate solution (1·5 per cent. ammonium molybdate in 0·005 N-sulphuric acid) adjusted to pH 4·0 with an equal volume of acetate buffer before use. Incubate at 37° for 10 minutes.

(4) Mount sections in slides, drain, and cover with 1 or 2 drops of ascorbic acid solution (2 per cent. ascorbic acid) adjusted to pH 4·0 with an equal volume of acetate buffer. Apply a coverslip and observe at once under the microscope.

Result

Inorganic phosphates appear blue.

METHOD FOR CARBONATES (after Bunting)

(*Formalin-fixed paraffin sections*)

The presence of gas bubbles arising from any deposit treated with dilute acid and examined under a coverslip is usually taken to indicate carbonate. Bunting (1951) described the following method (modified) for the positive identification of gas as CO_2.

Method

(1) Bring sections to acetone and allow to dry.

(2) Cover the selected area with clear mineral oil.

(3) Inject a microdrop of dilute acid (5 per cent. sulphuric) under the oil.
(4) If a gas bubble forms the acid drop is pushed aside and replaced by a drop of saturated barium hydroxide.

Result

The slow formation of a white precipitate at the interface between the barium solution and the gas bubble indicates CO_2 and therefore carbonate.

DITHIZONE METHOD FOR ZINC (after Mager *et al.*, 1953)

(*Freeze-dried, paraffin sections; cold knife or cold microtome, mounted sections*)

Stock Solutions

A. 0·01 per cent. dithizone in absolute acetone.
B. Complex-forming buffer. Dissolve 550 g. sodium thiosulphate (10 H_2O), 90 g. sodium acetate (3 H_2O) and 10 g KCN in 1 litre distilled water. Adjust pH to 5·5 with acetic acid and make up to 2 litres. Extract with dithizone in CCl_4 to remove traces of zinc.

Working Solutions

A. Dilute stock solution A 1·5 : 1 with distilled water.
B. Mix 24 ml. stock solution A with 18 ml. distilled water. Adjust to pH 3·7 with N-acetic acid. Add 5·8 ml. stock solution B and 0·2 ml. 20 per cent. sodium potassium tartrate.

Method

(1) Stain pairs of sections in A and B for 10 minutes.
(2) Wash in chloroform and rinse quickly in water.
(3) Mount in Karo syrup (p. 775).
Alternatively, for cold microtome sections, stain for 3 minutes in equal parts of stock tris buffer (pH 10) and 0·05 per cent. dithizone in absolute ethanol.

Result (Figs. 227 and 228)

A red or reddish-purple granular precipitate indicates the presence of zinc.

APPENDIX 25

PAS METHOD FOR DEXTRAN (after Mowry and Millikan, 1953)

(Cold ethanol; paraffin sections)

The original authors recommend that after 48 hours fixation in cold ethanol tissues should be transferred to fresh alcohol for 18 hours before clearing in light petroleum and embedding in paraffin.

Method

(1) Cut sections at 5μ and float sections on to clean slides moistened with 95 per cent. alcohol.

(2) Dry at 37° for a few hours and then at 56–60° for 20 minutes.

(3) Remove paraffin with xylene and transfer to absolute alcohol.

(4) Incubate with 1 per cent. periodic acid in 90 per cent. ethanol for 2 hours.

(5) Wash in 95 per cent. alcohol and then in 50 per cent. alcohol.

(6) Immerse in Schiff's reagent for 10 minutes.

(7) Wash in acid metabisulphite for 5 minutes.

(8) Dehydrate in alcohol, clear in xylene and mount in a suitable synthetic resin.

Result

Dextran and other polysaccharides stain magenta-red.

Control slides stained by the aqueous PAS method (Appendix 9, p. 832) should show loss of staining where this is due to dextran.

METHODS FOR DEXTRAN SULPHATE (Mowry, 1954)

(Cold ethanol; paraffin sections)

Method I

(1) Prepare sections as in the preceding method.

(2) Bring sections to 95 per cent. alcohol, containing 0·5 per cent. toluidine blue. Leave for 20 minutes.

(3) Transfer to 70 per cent. alcohol containing toluidine blue and then to 50 per cent. alcohol containing toluidine blue.

(4) Stain finally in 0·5 per cent. aqueous toluidine blue for 1–2 minutes.

(5) Rinse in 70 per cent. alcohol, dehydrate in 95 per cent. and absolute alcohol; clear and mount in a synthetic resin.

Method II

(1) Prepare sections as above.

(2) Stain in 0·005 per cent. toluidine blue in 70 per cent. alcohol for 5–10 minutes.

(3) Rinse in tertiary butyl alcohol.

(4) Clear in xylene and mount in a synthetic resin.

Result

Dextran sulphates stain (metachromatically) deep red.

IODINE METHOD FOR PVP (after Jancsó, 1954)

Method I

(1) Fix small pieces of tissue in a mixture containing 20 ml. saturated ammonium sulphate, 20 ml. saturated KIO_4 and 0·3 g. KI. Leave for 24 hours at room temperature, changing the fixative after 5 hours.

(2) Cut frozen sections, mount on slides in glycerine jelly containing enough Lugol's iodine to impart a brownish-yellow colour.

Method II

(1) Transfer tissues from stage 1 of Method I to 10 per cent. aqueous uranyl nitrate containing 1 drop per 10 ml. of Lugol's iodine. (The tissues should rest on cotton wool.) Leave for 24–36 hours.

(2) Transfer to a mixture of 3 parts alcohol and 7 parts xylene containing a small concentration of iodine.

(3) Transfer to xylene-alcohol 9:1 containing iodine as before.

(4) Embed in paraffin wax *in vacuo*.

(5) Cut sections 5–7 μ thick, remove wax with xylene and mount in Canada balsam.

Result

PVP stains brown, polyvinyl alcohol black.

CONGO RED METHOD FOR PVP (Freiman and Gall, 1955)

(*Formalin; paraffin sections*)

Method

(1) Bring sections to water.
(2) Stain in 2 per cent. Congo red in 10 per cent. ethanol for 30 minutes.
(3) Wash in water.
(4) Counterstain in 1 per cent. aqueous light green SF for 2 minutes.
(5) Rinse quickly.
(6) Blot and mount in Apathys gum syrup.

Result

PVP stains orange to cherry-red. Elastic tissue brown. Nuclei brown to green, other structures green.

CHLORAZOL FAST PINK METHOD FOR PVP

(*Formalin; paraffin sections*)

Method

(1) Bring sections to water.

(2) Stain in 1 per cent. Chlorazol fast pink in 50 per cent. ethanol at 60° for 10–30 minutes.

(3) Rinse in water.
(4) Counterstain nuclei with Mayer's hæmalum, 3 minutes.
(5) Wash, differentiate in 1 per cent. acid alcohol.
(6) Wash in water until nuclei are blue.
(7) Dehydrate, clear and mount in a synthetic resin.

Result (Fig. 229)

PVP stains pink to cherry-red; nuclei blue.

XANTHYDROL METHOD FOR UREA

Method

(1) Fix thin blocks (1–2 mm.) of tissue for 3–6 hours in 5 per cent. xanthydrol in acetic-ethanol (Acetic acid 60 ml.; ethanol 40 ml.) at 56–60°.
(2) Wash in water for 1 hour.
(3) Fix in 10 per cent. formalin, 6 hours.
(4) Wash briefly in water.
(5) Cut frozen sections 5–10 μ thick and mount on slides.
(6) Blot dry and transfer to 95 per cent. alcohol.
(7) Cover with a thin film of 1 per cent. celloidin in alcohol-ether.
(8) Stain briefly in 1 per cent. eosin and wash.
(9) Dehydrate, clear and mount in balsam.
(10) Examine under ordinary and polarized light.

Result

Yellowish-brown birefringent, crystals of urea-xanthydrol indicate sites where urea was present.

HEXAMINE SILVER METHOD FOR URIC ACID

(*Alcohol; paraffin sections*)

Method

(1) Fix fresh tissues in absolute alcohol for 24 hours, changing the alcohol at least once during this time.
(2) Embed in paraffin. Cut sections 5–7 μ thick and float on to slides with 95 per cent. alcohol.
(3) Dry at 37° for 2 hours.
(4) Remove wax with light petroleum and wash in absolute alcohol.
(5) Transfer directly to hexamine-silver solution (Appendix 23, p. 919) at 37°. The slides should remain in this solution until uric acid (urate) deposits are black (15–60 minutes).
(6) Rinse in 5 per cent. sodium thiosulphate, 3 minutes.
(7) Counterstain with 1 per cent. eosin, 1 minute.
(8) Wash. Dehydrate, clear and mount in a synthetic resin.

Result

Urates black. Calcium phosphate and carbonate deposits will also stain black if large enough to withstand solution in the silver bath.

APPENDIX 26

ACRIDINE ORANGE METHOD FOR NUCLEIC ACIDS

(after von Bertalanffy and Bickis)

(Fresh smears; fresh frozen, cold microtome sections mounted on coverslips)
(Both types of preparation may be post-fixed in acetic-ethanol)

Stock Solutions

Acridine Orange. 1:1000 solution in distilled water.
Krebs-Ringer (Stock)

80 parts 0·9 per cent. NaCl (0·154 M)
4 parts 1·15 per cent. KCl (0·154 M)
3 parts 0·11 M-$CaCl_2$
1 part 2·11 per cent. KH_2PO_4 (0·154 M)
1 part 3·82 per cent. $MgSO_4.7H_20$.

This solution can be kept at 0–4°.

For use the stock medium is adjusted to the chosen pH (6·0 to 6·5) with 0·1 M-sodium phosphate buffer. One part of stock acridine orange solution is then diluted with 9 parts of the adjusted Krebs-Ringer solution.

Method

(1) Cover sections with the staining solution for 15 minutes or longer, at room temperature.

(2) Remove staining solution with filter paper and cover section with buffered Krebs-Ringer solution. Apply a coverslip or mount on a slide (whichever applies) and examine by fluorescence microscopy using blue light.

Result

Nuclei (DNA) green. Nucleoli and cytoplasmic nucleic acids (RNA) red. Fibrous connective tissue, green.

SELECTIVE STAIN FOR MUCINS (after Hicks and Matthaei)

(Paraffin sections ; formalin or special fixation)

Method

(1) Bring sections to water.
(2) Mordant with 4 per cent. iron alum for 5–10 minutes.
(3) Wash in running water 2–3 minutes.
(4) Stain in 1 : 1000 aqueous acridine orange for 2 minutes.
(5) Wash briefly and mount in glycerine.
(6) Examine by fluorescence microscopy using a blue light and yellow eyepiece filter.

Result

Acid mucopolysaccharides fluoresce reddish orange. The fluorescence of most other tissue components is quenched.

PREPARATION OF FLUORESCEIN ISOTHIOCYANATE I (after Riggs *et al.*)

Fluorescein *Iso*thiocyanate I

Aminofluorescein diacetate I is prepared according to the directions given in Appendix 6, p. 810. Dissolve 5 g. of this compound in 20 ml. of acetone and add, drop by drop, to 4 ml. of thiophosgene in 20 ml. acetone at 40°, with constant stirring. Heat under reflux for 4 hours. After removal of solvent and excess thiophosgene under reduced pressure the residue is dissolved in 20 ml. acetone and added dropwise to 1200 ml. light petroleum (B.P. 60° to 70°). The orange precipitate is collected, washed with light petroleum and dried. (Yield, 86 per cent.).

PREPARATION OF RHODAMINE B ISOTHIOCYANATE (after Riggs *et al.*)

4–Nitrophthalic Anhydride

Heat 4-nitrophthalic acid for 2 hours at 170–175°. After cooling, the light tan solid is powdered. (M.P. 115–117°.)

Nitrorhodamine B

Suspend 4·8 g. finely powdered 4-nitrophthalic anhydride in 100 ml. xylene and heat with vigorous stirring to reflux temperature. Maintain the mixture at just below this temperature and add 8·2 g. *m*-diethylaminophenol in 50 ml. xylene, drop by drop over a period of 30 minutes. The solution should be red at this stage. Maintain at reflux temperature for 18 hours ; discontinue stirring after 2 hours. Distil the dark purple solution under reduced pressure until the volume reaches 30 ml. Cool the mixture. A thick gum forms. Decant the supernatant and wash the gum with fresh xylene. Dissolve the gum in 200 ml. boiling chloroform. Filter, cool and pour rapidly into 800 ml. cold light petroleum (B.P. 60° to 70°). Wash the precipitated solid with this solvent and dry. Magenta powder. (Yield, 75 per cent.)

Aminorhodamine B

Suspend 2 g. nitrorhodamine B and 1 g. Raney nickel in 200 ml. ethanol. Hydrogenate at 50 pounds pressure and 35 for 16 hours. Filter the solution to remove the catalyst. Evaporate to 3 ml. and pour into 400 ml. cold light petroleum (B.P. 60° to 70°). Collect precipitate, wash and dry. (Yield, 78 per cent.)

Rhodamine B *Iso*cyanate

Suspend 1 g. aminorhodamine B in 15 ml. acetone and add drop by drop to 1 g. thiophosgene in 5 ml. in a 50 ml. Erlenmeyer flask equipped with a magnetic stirrer. Stir at 40° for 8 hours, cool, filter and concentrate the filtrate to a gum. Redissolve the gum in 5 ml. acetone and pour into 200 ml. light petroleum (B.P. 60–70°). A magenta precipitate is produced. Filter, wash and dry. (Yield, 85 per cent.)

PROTEIN CONJUGATION PROCEDURE WITH ISOTHIOCYANATES

Place 10 ml. 0·85 per cent. saline, 3 ml. carbonate-bicarbonate buffer (0·5 M, pH 9·0) and 2 ml. reagent grade acetone in a 50 ml. Erlenmeyer

flask equipped with a mechanical stirrer. Cool to 0° and add, with constant stirring, 10 ml. of the dilute protein solution (of known strength). Cool again until ice crystals form. Slowly add 1·5 ml. acetone containing the required amount of dye (0·05 mg. per mg. protein). Transfer to a cold room (4) and continue stirring for 18 hours. Dialyse against phosphate buffered saline (0.01 M, pH 7·2) until the dialysate is non-fluorescent when irradiated with ultraviolet light.

Before use, all conjugates must be adsorbed with dried mouse liver powder, as described in Appendix 6, p. 811.

MERCURY VAPOUR LAMPS FOR FLUORESCENCE MICROSCOPY

The lamps listed below have all been used successfully as sources of ultraviolet light for fluorescence microscopy.

B.T.H. (British Thompson Houston)	Mercra Lamp (150 watts)
General Electric	A H 4 (100 watts)
General Electric	A H 6 (1000 watts)
Gesellschaft-Hanau . . .	Quartzlampen S.81
Hanovia	Mercury Vapour Lamp (240 watts)
Mazda	ME/D (250 watts)
Osram (Reichert)	HBC200 (200 watts)
Philips (Leitz)	C S 150 (150 watts)

FILTERS (Ultraviolet-Transmitting)

*Woods Glass (+ 5 per cent. $CuSO_4$ solution).
Corning 7–37, Violet Ultra.
B G 12 Schott, Jena (Long UV and blue light).

FILTERS (Ultraviolet-Absorbing)

Euphos Leitz.
Minus Blue Ilford.
O.G.1 Schott, Jena.
O.G.5 Schott, Jena.
Corning 3–75 (3060 glass), Noviol O.
Polaroid UV 395.
*Wratten 2 B.
Wratten 2 A.

* A combination of these two is recommended for fluorescent microscopy and photomicrography.

APPENDIX 27

STRIPPING FILM TECHNIQUE FOR AUTORADIOGRAPHY (Pelc)

(Freeze-dried or fresh frozen sections, alcohol-fixed. Paraffin sections after alcohol)

Special stripping film for autoradiography is obtainable from Kodak Ltd., Harrow-Wealdstone, Middlesex, England. The film as delivered is mounted on glass and consists of two layers—10 μ thick gelatine facing the glass with a 4 μ thick photographic emulsion upon it. The emulsion is very sensitive to scratching and at all stages careful handling is neeessary. Storage in a cold room or refrigerator at 4° is advisable. Low baekground is essential to the method and the film must be kept in the shade, even from the recommended dark light, when not actually handled.

Full consideration of the various types of emulsion and details for the photographic processing of these are given by Fitzgerald (1955).

Method

(I) Preparation of Sections. (1) Fix small pieees of tissue in absolute alcohol for 1–3 hours.

(2) Clear in benzene, 15 minutes, followed by warm benzene (56°), 15 minutes.

(3) Transfer to four changes of molten paraffin wax (56°), for 30 minutes in each case.

(4) Embed, block and cut sections at 5 μ or less and float out on warm water.

(5) Pick up sections on to slides which have previously been dipped in an aqueous solution of 1 per cent. gelatin and 0·1 per cent. ehrome alum, and then allowed to dry.

(II) Application of Film (Fig. 245). (1) Cut film in squares with seissors, razor blade or scalpel, allowing for a generous overlap over the edge of the slide ; $\frac{1}{4}$ to $\frac{1}{2}$ inch from the edge of the slide should be discarded. Leave in the dark for 5–10 minutes.

(2) Strip one square slowly, starting at one corner and float on dust-free, fresh distilled water at 23°–25°, emulsion-side *downwards* for 2–3 minutes. (The water should only be used for one run of eoatings.)

(3) Place a deparaffinized slide in the water, section or squash faeing upwards, and lift out of the water so that the film covers the speeimen.

(4) Dry in front of a fan or hair-dryer at room temperature.

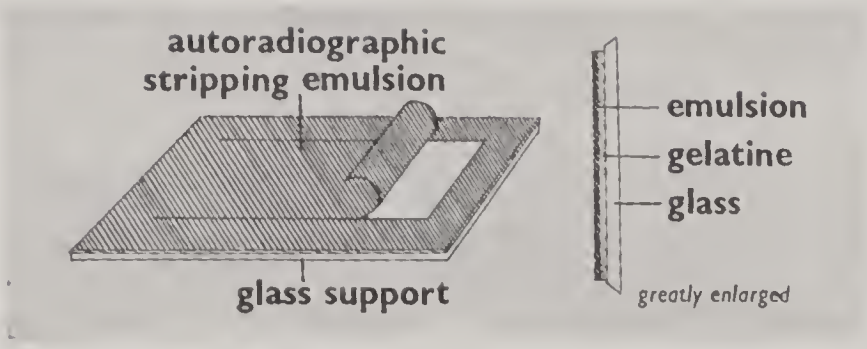
(5) Store in the dark for exposure. (Some workers expose at room temperature, others at 4°. Warm rooms should be avoided.)

(6) Photographic processing should be carried out aeeording to the maker's instructions. Use chemieals of analytical purity dissolved in distilled water and filtered. All processing should be done at 17·5 ± 0·5°.

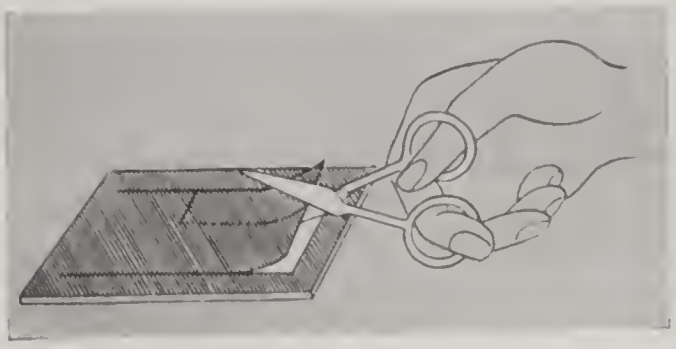
(7) Rinse in distilled water, and in running tap-water if this is suffieiently clear and cool, for 1 hour. Otherwise use 4–6 changes in distilled water.

(8a) For phase contrast mieroscopy mount in glyeerine jelly ; glychrogel (Appendix 1) is suitable for this purpose.

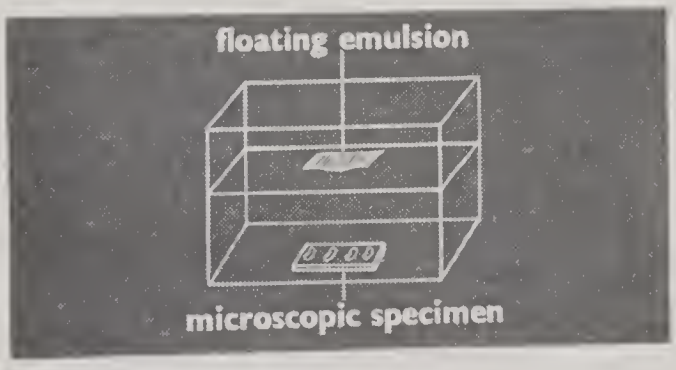
(8b) Staining with hæmalum : plaee moist preparation in eelestin blue



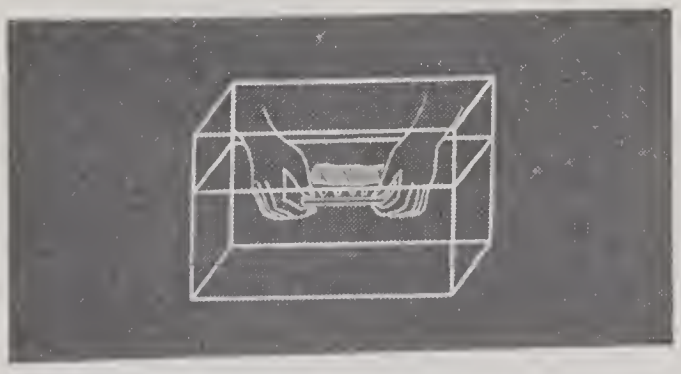
1. Cut with knife and strip film as shown.



2. Cut off the area of the emulsion required (usually 1 in. square).



3. (*a*) Place microscopic specimen on bottom of dish filled with distilled water.
(*b*) Turn emulsion downwards and float on surface of distilled water (23°–25°) for approximately 5 minutes.



4. Lift specimen to contact emulsion until the latter covers the specimen.

Courtesy R. H. Hertz & Kodak, Ltd.

5. Dry in dark and expose.

6. Process emulsion at 17·5 ± 0·5° whilst superimposed on specimen.

Fig. 245. Method of autoradiography after Pele.

(see PAS technique, Appendix 9) for 5 minutes, wash briefly and immerse in Mayer's hæmalum, 5 minutes.

(9) Differentiate in 1 per cent. aqueous HCl for 15–60 seconds, or until there is minimal staining of the gelatine compatible with retention of nuclear staining.

As an alternative, deparaffinized preparations can be stained before stage 3, by the Feulgen reaction (Appendix 8) or with Neutral red-Carbol fuchsin (30 ml. 2 per cent. Neutral red with 10 ml. Ziehl-Neelsen's carbol fuchsin). Hæmatoxylin may also be used if hardener and fixer without acetic acid are used in processing. Objections have been made to the use of iron hæmatoxylins for use at stage 8b since silver is removed by iron salts, especially in acid solution. Removal of silver does not apparently occur with the iron alum-mordanted celestin blue solution recommended above.

CONTACT METHOD FOR AUTORADIOGRAPHY (Pelc)

(*For discussion of the technique of contact autoradiography see* Lotz et al., 1952)

Method

(1) Prepare specimen as in Stages 1–5 of the previous method.

(2) Remove wax by immersion in light petroleum.

(3) Bring the section into contact with X-ray film (for highest speed) or fine-grained film (for better resolving power).*

(4) Apply even pressure by putting film and specimen into a printing frame, X-ray cassette, or simply by putting a weight on it.

(5) Store in the dark for exposure.

(6) Process the film according to the maker's instructions, paying particularly strict attention to cleanliness throughout.

COLD MICROTOME SECTIONS FOR AUTORADIOGRAPHY

The use of cold knife sections for autoradiography was introduced by Witten and Holmström (1953). This method is particularly applicable to cold microtome sections if the cryostat can be housed in the dark room.

Method

Two alternative methods can be employed :

(1) The section may be picked up from the knife on a clean slide by means of the suction pick-up described in Appendix 2, p. 784. Thereafter it may be fixed, if necessary, in the appropriate fixative. After drying, stripping film can be applied directly.

(2) The section may be picked up directly on contact film of suitable type, using the suction pick-up. It can then be exposed, and developed, without further treatment.

* Film is preferable to plate because of the unevenness of the latter. If necessary, a thin film of cellophane (less than 0·2 mm. thick) can be inserted between the film and the specimen.

AUTORADIOGRAPHY OF MUCOPOLYSACCHARIDES (after Heatley *et al.*, 1956)

Method of Fixation

(1) Fix tissues immediately after removal in the following mixture

" Stock " Phosphomolybdic acid (25 per cent. in 6·25 N HCl)	20 ml.
Ethanol	65 ml.
40 per cent. Formaldehyde	10 ml.
Water	5 ml.

This should be freshly prepared from stock phosphomolybdic acid on the day of use.

(2) After 8–17 hours at room temperature transfer the blocks to 70 per cent. alcohol. A greenish colour in the tissues may be ignored.

(3) Dehydrate, clear and embed.

The authors recommend embedding in ester wax (Steedman, 1947), or in a modified ester wax having the following composition.

Diethylene glycol monostearate, purified	100 g.
Diethylene glycol distearate	100 g.
Castor Oil	10 ml.

Melt at 50° and filter.

Autoradiography

(1) Mount sections on slides with egg albumin, dry overnight at 37°, dewax and take down to water.

(2) Immerse in 2 per cent. sodium sulphate for 3 hours. This removes any unbound $^{35}SO_4$.

(3) Wash in water for 15 minutes.

(4) Stain by the PAS procedure (Appendix 9, p. 832) with counterstains if required.

(5) Dry the slide and cover with 0·5 per cent. celloidin.

(6) Rinse in distilled water.

(7) Transfer to dark room and attach Kodak stripping film to the section.

(8) Place slides in a drying box till dry (1 to 1½ hours).

(9) Transfer slides to light-tight, metal-lined boxes and place in a refrigerator at 0°–4° for the required period of exposure (2–12 weeks with ^{35}S).

(10) Develop in Kodak D 196 developer.

(11) Fix and wash in tap water for 30–60 minutes.

(12) Rinse in distilled water and dry completely in the drying box.

(13) Remove overlapping film at the back of the slide.

(14) Immerse in 60 per cent. alcohol, absolute alcohol, and finally in xylene.

(15) Mount in Canada Balsam or other suitable medium.

Result

The results are shown in Plates XC, XD and XE.

MOLECULAR WEIGHT AND SOURCE LIST

Compound	M.W.	Source *
Acetaldehyde	44·23	—
Acetic acid	60	—
Acetic anhydride	102·05	—
Acetobromoglucose	411	LL
Acetophenone	120·66	—
p-Acetoxydiphenyl	211	—
2-Acetoxy-3-naphthoic anilide	321	DJ
2-Acetoxy-3-naphthoic-*o*-anisidide	352	—
2-Acetoxy-3-naphthoic-*o*-toluidide	336	—
N-Acetyl-DL-alanine	147	SIG; GG
O-Acetyl-5-bromoindoxyl	254	GG; DJ
O-Acetyl-4-chloro-5-bromoindoxyl	289	GG
Acetyl chloride	78·5	LL; HW
Acetyl choline chloride	181·7	R; DJ
Acetyl-D-glucosamine	237	LL
N-Acetyl-DL-leucine	190	SIG; GG
Acetylthiocholine iodide	288	LL; DJ
cis-Aconitic acid anhydride	174	CC; LL
Adenosine diphosphate	427·2	SIG; LL; GG
Adenosine-5-phosphate (Adenylic acid)	347·2	SIG; LL; GG; DJ
Adenosine triphosphate	507·2	SIG; GG; LL; DJ
Adrenalin hydrochloride	217·5	AH
Adrenochrome	179·17	NB
Alanyl-β-naphthylamide	—	DJ
Albumin (egg), Cryst.	—	AR
Alcian blue	—	ICI; DJ; GG
Alginic acid	(176)n	LL
Alloxan	160·09	LL; BDH; GN
Aluminon (ammonium aurine tricarboxylate)	473·45	BDH; MB
Aluminium potassium sulphate (Potash alum) ($12H_2O$)	474·39	—
Aminoacetic acid (Glycine)	75·07	HW
5-Aminoacridine hydrochloride	230·5	MB
2-Aminoanthraquinone	223·2	BDH
p-Aminodiphenyl	211	—
2-Amino-2(hydroxymethyl)1,3-propanediol (Tris)	121·2	EK; BDH; LL; CC
8-Amino-1-naphthol-3,6-disulphonic acid (H-acid)	319·3	BDH
8-Amino-1-naphthol-5-sulphonic acid (S-acid)	239·25	EK
p-Aminophenyl arsonic acid	216·9	BDH
o-Amino-phthalic cyclic hydrazide	177·2	BDH
6-Aminopurine sulphate	404·4	SIG; GG
3-Amino-1,2,4-tetrazole	84	LL
2-Aminothiazole	100	LL
Amobarbital (Amytal)	226·3	—
Amobarbital sodium (Sodium Amytal)	248·3	—
α-Amylase	—	NB; LL

* See List of Sources, p. 966.

Compound	M.W.	Source *
β-Amylase	—	NB; LL
iso-Amyl nitrite	117·15	BDH
L-Ascorbic acid	176·12	R; LL
L-Asparagine	132·12	CC; DJ
Auramine O	303·8	GG; DJ
Benzaldehyde phenylhydrazone	196·2	BDH; DJ
Benzene sulphohydroxamic acid	152	HW
Benzidine dihydrochloride	257·16	LL
Benzoin	212	BDH
Benzoquinone	108·1	BDH
Benzoyl chloride	140·57	—
6-Benzoyl-2-naphthyl phosphate (sodium)	—	DJ
6-Benzoyl-2-naphthyl sulphate (potassium)	—	BL
Berberine sulphate	433·4	BDH
Blue tetrazolium (BT)	727·67	GG; DJ; BDH
Boric acid	61·84	—
5-Bromoindoxyl acetate	254	GG; DJ
6-Bromo-2-naphthol	223·07	DJ
6-Bromo-2-naphthyl glucoside	—	DJ
6-Bromo-2-naphthyl glucuronide	—	DJ
Bromosuccinimide	178	HW
N-Butyl alcohol	74·12	—
Butyrylthiocholine iodide	224	LL; DJ
Carbazole	167·2	LL
Carboxypeptidase	—	AR; LL
Casein	—	LL; BDH
Catalase	225,000	B; SIG; LL
Cetylpyridinium chloride	358	BDH
Chloral hydrate	165·4	—
p-Chloroanilidophosphonic acid	206·5	LL; CC
1-Chloro-2,4-dinitrobenzene	202·6	BDH
p-Chloromercuribenzoic acid	477·3	—
p-Chloromercuriphenylsulphonic acid	394	SIG; LL
1-(4-Chloromercuriphenylazo-2-naphthol)	482	LL; BIOS
Cholesterol	386·6	HW
Cholesteryl acetate	428·7	BDH
Cholesteryl oleate	651·12	BDH
Choline chloride	139·63	BDH
Chromyl chloride	154·92	HW
Chymotrypsin	—	BIOS; AR; LL; SIG
Cobaltous acetate ($4H_2O$)	249·1	—
Cocarboxylase	478	LL; R; SCH
p-Cyanoaniline	118·13	GN
Cysteic acid	169·2	LL
L-Cysteine	121·16	LL
Cystine	240·31	—
Cytochrome C	13,000	BIOS; LL; B
62.C.47	608	W
Desoxycholic acid	392·6	GN
Diacetin	176·2	BDH

* See List of Sources, p. 966.

Compound	M.W.	Source *
m-Diacetoxybenzene	135	BDH
Diastase	—	BDH
1-Diazo-2-naphthol-4-sulphonic acid	251	LL
2,7-Diaminofluorene	196·21	LL
2,4-Dibromo-1-naphthol	302	BDH
2,6-Dichloroquinone-chloroimine (Gibbs' reagent)	210	BDH; DJ
Dichloro-difluoromethane (Arcton 6, Freon 12)	120·92	ICI
Dichlorohydrin (1,3-dichloropropanol)	129	BDH
2,4-Dichloro-1-naphthol	213	BDH
2,6-Dichlorophenol indophenol	290·1	LL; BDH
m-Diethylaminophenol	165·24	EK; BDH
Diethylene dioxide (Dioxan)	88·1	BDH
Diethylene glycol monostearate	—	WC
Diethylene glycol distearate	—	BDH
Diethyl-*p*-nitrophenyl phosphate (E 600)	259·2	LL
Digitonin	125·3	LL; BDH
2,4-Dihydroxybenzaldehyde	138·12	BDH
2,2′-Dihydroxy-6,6′-dinaphthyl disulphide (DDD)	—	DJ; BL
4,5-Dihydroxy-2,7-naphthalene disulphonic acid	356·33	EK
DL-3,4-Dihydroxyphenyl alanine	197·2	LL; BDH
Dimedone	140·2	BDH
p-Dimethylaminobenzaldehyde	149·2	HW; BDH; LL
p-N,N-Dimethylaminocinnamaldehyde	—	AL
Dimethyl formamide	73	LL; BDH
Dimethyl glyoxime	116·12	HW
3,3′-Dimethylnaphthidine	312	LL; BDH
Dimethyl-*p*-phenylenediamine hydrochloride	209·13	BDH
4,5-Dimethylthiazolyl tetrazolium bromide	415	GG
2,3-Dimercaptopropanol (BAL)	124·2	LL; BDH
Dinitrofluorobenzene	186	BDH; DJ
2,4-Dinitrophenylhydrazine	198·14	BDH
2,4-Dinitrophenol	184·11	BDH
2,4-Dinitroresorcinol	200·1	BDH
Diphenyl carbazide	242·3	HW; MB
sym-Diphenyl carbazone	240·26	LL
2,5-Diphenyl-3,4-distyrylphenyl tetrazolium chloride	586	MB
Diphenylthiocarbazone	256·3	LL; HW; MB
Diphosphopyridine nucleotide (DPN)	663·44	B; CC; SCH; SIG
Diphosphopyridine nucleotide, reduced (DPNH)	—	B; CC; SIG
Di-*iso* propyl Fluorophosphonate (DFP)	174·15	LL
Di-*iso* propyl phosphorodiamidic fluoride	182	LL
2,2-Diquinolyl	395·5	LL
Eserine	275·36	BDH
Eserine sulphate	648	BIOS
Ethopropazine hydrochloride	312·5	MB
Ethylenediamine tetra-acetic acid (Disodium)	372·2	LL; HL
N-Ethyl maleimide	125	LL; DJ
Ethyl phenyl carbamate	165·2	BDH

* See List of Sources, p. 966.

Compound	M.W.	Source *
Fast Black K salt (*p*-nitroaniline-azo-2,5-dimethoxyaniline)	—	GG
Fast Blue B salt (*o*-dianisidine)	—	DJ
Fast Blue BB salt (4-benzoylamino-2,5-diethoxyaniline)	—	GG; DJ
Fast Blue RR salt (4-benzoylamino-2,5-dimethoxyaniline)	—	DJ; GG
Fast Blue VB salt (4-amino-4′-methoxydiphenylamine)	—	DJ; GG
Fast Garnet GBC salt (*o*-amino-azo-toluene)	—	DJ; GG
Fast Red AL salt (1-aminoanthraquinone)	—	DJ; GG
Fast Red B salt (5-nitro-*o*-anisidine)	—	GG
Fast Red GG salt (*p*-nitroaniline)	—	GG
Fast Red GL salt (3-nitro-*p*-toluidine)	—	GG
Fast Red 3GL salt (4-chloro-2-nitroaniline)	—	GG
Fast Red LTR salt (2-methoxy-5-diethylamino sulphaniline)	—	GG
Fast Red RC salt (4-chloro-*o*-anisidine)	—	DJ
Fast Red RL salt (3-nitro-*o*-toluidine)	—	GG
Fast Red TR salt (5-chloro-*o*-toluidine)	—	DJ; GG
Fast Scarlet GG salt (2,5-dichloroaniline)	—	GG
Fast Scarlet R salt (4-nitro-*o*-anisidine)	—	GG
Fast violet B salt (2-benzoylamino-4-methoxy-toluidine)	—	GG
Fluorescein amine (I or II)	—	LL; DJ
1-fluoro-2,4-dinitrobenzene	182	LL
Fluorescein *iso*thiocyanate	—	DJ
Fructose-,1,6-diphosphate (disodium)	340·13	LL; DJ
Fumaric acid	116·08	BDH; LL
D-Galacturonic acid	194·14	LL; DJ
D-Galactose-1-phosphate (barium)	274·16	DJ
Glucose pentaacetate	390·34	LL
D-Glucosamine hydrochloride	215·64	DJ; BDH
β-Glucosidase	—	SIG; LL
1,5-Gluconolactone	178·15	BDH
Glucose-1-phosphate (dipotassium)	372·36	SIG; BDH; B
Glucose-6-phosphate (disodium)	304·11	SIG; BDH
L-Glutamine	146·15	DJ; BDH
Glutamic dehydrogenase	—	B; SIG
Glutathione	307·33	SIG; DJ; GG; B
Glycine	75·07	BDH; HW
D-Glyceraldehyde	90·1	LL
Glyoxylic acid	74·04	BDH
Glyoxal sodium bisulphite	145	BDH
Guanine	151·13	SIG
Guaiacol	124·14	HW; BDH
Hæmin	651·9	LL
Hexamethylene-tetramine (Hexamine)	140·19	BDH
Hexokinase	—	SIG
Hydrazine dihydrochloride	104·98	BDH
Hydrazine hydrate	50·06	BDH

* See List of Sources, p. 966.

Compound	M.W.	Source *
Hyaluronidase	—	SIG; LL
1-Hydroxy-2-acetonaphthone	—	EK
DL-α-Hydroxybutyric acid	104·1	LL; CC
DL-β-Hydroxybutyric acid	104·1	BDH; LL; HW; CC
Hydroxylamine hydrochloride	69·5	BDH
3-Hydroxy-2-methyl-1,4-naphthoquinone (Phthiocol)	187·2	LL
2-Hydroxy-3-naphthaldehyde	172·19	DJ; CC
1-Hydroxy-2-naphthoic acid	188·17	LL; EK
2-Hydroxy-3-naphthoic acid	188·17	LL
2-Hydroxy-3-naphthoic acid hydrazide	246	DJ; CC; LL; BL
8-Hydroxyquinoline glucuronide	338·3	LL; CC
8-Hydroxyquinoline sulphate	388·4	BDH
5-Hydroxytryptamine (Creatinine sulphate)	—	SIG; R
DL-5-Hydroxytryptophan	—	LL; CC; SIG
Indole	117·14	LL
3-Indolyl aldehyde	131	LL
Indoxyl acetate	175·2	DJ; LL; CC
Indoxyl butyrate	203·2	CC
Iodoacetic acid	185·96	BDH; DJ
Iodoacetamidonaphthol	—	DJ
2-(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride (INT)	507·7	CC; DJ; LL
*Iso*citric acid (Trisodium)	276·12	SIG
Isoprene	68·11	LL
Janus Green B	—	GG; DJ
α-Ketoglutaric acid	146·1	SIG; CC
Lactic acid dehydrogenase	100′000	LL; SIG
Laurent's acid	223·24	BDH
D-Leucine	131·2	CC; LL
L-Leucyl-β-naphthylamide	—	DJ; CC
2,6-Lutidine	107·15	BDH
Maleic acid	116·08	BDH; LL
L-Malic acid	134·09	CC; EK
Malic dehydrogenase	—	SIG
Manganese chloride	197·92	BDH; HW
Menaphthone	172·2	LL
Mercury orange	482	LL; BIOS
DL-Methionine	149·2	CC; SIG; LL
o-Methoxyphenol	124·14	HW; BDH
DL-Methoxyphenylacetic acid	166	EK
Methylene blue (zinc free)	374	BDH; DJ
4-Methyl-6-chlorothionaphthene	182	ICI; DP
β-Methylindole	131·2	CC
9-Methyl-2,6,7-trihydroxyfluorone	228·2	BIOS; LL
4-Methyl umbelliferone	177·15	BDH
Mipafox	182	LL
Morin (2′,3,4′,5,7-Pentahydroxyflavone)	302·23	LL; MB

* See List of Sources, p. 966.

Compound	M.W.	Source *
Myristic acid	228·4	BDH
Myristoyl choline chloride	365	DJ; LL
1-Naphthalene acetamide	185·3	LL
1,2-Naphthoquinone	158·15	LL
1-Naphthoxyacetic acid	202	HW
1-Naphthyl acetate	186·21	BDH; DJ
1-Naphthyl acetyl-β-glucosamine	354	—
1-Naphthyl *iso*cyanate	169·2	BDH
1-Naphthyl-β-glucuronide	300	—
1-Naphthyl phosphate (sodium)	247	HW; DJ; CC
1-Naphthylamine	143·18	—
2-Naphthyl acetate	186·21	BDH; DJ; CC
2-Naphthyl laurate	326·41	DJ; CC
2-Naphthyl myristate	354·52	DJ; CC
2-Naphthyl stearate	410·63	DJ; CC
Neotetrazolium chloride (NT)	645	LL; NB; CC; DJ
Nicotinamide	122·12	SIG; DJ; CC; LL
Ninhydrin	178·14	LL; DJ; CC
p-Nitrobenzaldehyde	151·12	LL; DJ
Nitro blue tetrazolium chloride (Nitro-BT)	851	DJ; GG
4-Nitrophthalic acid	211·14	EK; BDH
1-Nitroso-2-naphthol	173·2	BDH
1-Nitroso resorcinol	139·11	BDH
Noradrenaline bitartrate	317	LL
iso-OMPA	342	LL
Orcinol	—	DJ; CC; GG
Oxalic acid	126·07	—
Oxalacetic acid	132·18	LL; CC; SIG
Oxalyl chloride	126·93	BDH
Papain	—	SIG; LL
Pectinase	—	SIG; ER; LL
Pepsin	—	SIG; AR; LL
Peptidase	—	LL; AR; SIG
Peracetic acid (40 per cent.)	76·05	LA
Perchloric acid	100·5	BDH
Periodic acid ($2H_2O$)	227·96	DJ; BDH; HL
p-Phenetidine hydrochloride	172·7	HW
DL-Phenylalanine	165·2	BDH
Phenylhydrazine hydrochloride	144·61	BDH
α-Phenyl indole	193·14	LL
Phenyl *iso*cyanate	119·12	BDH
Phenyl mercuric chloride	313·18	BDH
1-Phenyl-3-(M-Nitrobenzamidopyrazolone)	—	EK
N-Phenyl-*p*-phenylene diamine	202	EK
β-Phenyl-propionic acid	150·2	HW
1-Phenyl semicarbazide	151·17	GN
9-Phenyl-2,3,7-trihydroxyfluorone	290·21	HW
Phthalic anhydride	148·11	BDH
Phosphatase, alkaline	—	AR; SIG
6-Phosphogluconate (Barium)	430	SIG
Phosphorylase	—	SIG

* See List of Sources, p. 966.

Compound	M.W.	Source *
Polyvinyl pyrrolidone	11′000	LL
Potassium-6-benzoyl-2-naphthyl sulphate	—	DJ
Potassium-6-bromo-2-naphthyl sulphate	—	DJ
Potassium hydrogen saccharate	248	BDH
Potassium dihydrogen phosphate	136·09	BDH; HW
Potassium ferricyanide	329·26	BDH; HW
Potassium ferrocyanide	422·21	BDH; HW
Proflavine hemisulphate	552·62	BDH
Propane-1,2-diol	76·10	BDH
Propiolactone	—	LL
Propionic acid	74·08	BDH; HW
Propionyl choline chloride	212	DJ
Pyridine	79·10	BDH
Pyridoxal-5-phosphate (Codecarboxylase)	247·15	BIOS
Pyrocatechol	110·11	BDH
Quinalizarin	272·2	LL
Quinolyl-8-glucuronide	338·3	LL; CC
Rancy nickel	—	BDH
Resorcinol	110·11	—
Rhodizonic acid (Sodium salt)	214·05	BDH
Ribonuclease	—	LL; AR; SIG
Ribose-5-phosphate (Barium salt)	366	LL; SCH
Rubeanic acid (Dithiooxamide)	120	GN; BDH
Rubidium trichloride	207·47	BDH
Salicylaldehyde	122·12	LL; BDH
Semicarbazide hydrochloride	111·54	BDH; LL
Semicarbazide nitrate	138·09	BDH
Serotonin creatinine sulphate	405	LL; CC; R
Sinigrin	415·48	CC
Sodium acetate ($3H_2O$)	136·09	—
Sodium azide	65·02	LL
Sodium barbitone	206·18	BDH
Sodium bicarbonate	84	—
Sodium carbonate	106	—
Sodium citrate ($2H_2O$)	294·11	HW; BDH; MB
Sodium DL-*iso*citrate	258	CC
Sodium cyanide	49·02	—
Sodium diethyl dithiocarbamate	171·27	BDH; MB
Sodium dihydrogen orthophosphate ($2H_2O$)	156·01	BDH
Sodium fluoride	42·0	—
Sodium formate	68·02	—
Sodium L-glutamate	169	LL
Sodium α-glycerophosphate	—	—
Sodium β-glycerophosphate ($5\frac{1}{2}H_2O$)	315·15	LL
Sodium hydrogen malate	155·08	BDH
Sodium hydrogen citrate	263·12	BDH
di-Sodium hydrogen orthophosphate	141·97	BDH
di-Sodium hydrogen orthophosphate ($12H_2O$)	358·16	BDH
Sodium hydrosulphite (dithionite)	174·13	BDH
Sodium iodoacetate	180·1	LL

* See List of Sources, p. 966.

Compound	M.W.	Source *
Sodium α-naphthyl acid phosphate	—	HW; DJ; LL
Sodium nitroprusside	297·97	—
Sodium phenyl phosphate	254·10	BDH
Sodium oxalate	134	—
Sodium rhodizonate	214·05	LL
Sodium succinate	270·16	—
Sodium D-tartrate	230·09	BDH
Sodium taurocholate	—	BDH; LL
Sodium tetraborate	201·3	—
Sodium thioglycollate	114·11	LL; BDH
Sodium thiosulphate ($5H_2O$)	248·19	—
Solochrome cyanine R	—	ICI; BDH; GG
Sulphathiazole	255·32	MB; CC
D-Tartaric acid	150·09	BDH
L-Tartaric acid	—	BDH; LL
Terephthalaldehyde	134·13	GN
α-α′-Terephthaloyl-bis-(5-chloro-2,4-dimethoxy acetanilide)	—	PF
Tetraethyl ammonium hydroxide	147	BDH
Tetraethyl pyrophosphate (TEPP)	290·20	LL
Tetrahydrofuran	72·11	LL; BDH
Tetramethyldiaminotriphenyl methane	330	HW
Tetra*iso*propylpyrophosphoramide (*iso*-OMPA)	342	LL
Thioacetamide	75·14	BDH
Thioacetic acid (Thiolacetic acid)	76·12	BDH
Thiobarbituric acid	144·16	GN; BDH
Thioglycollic acid	92·12	BDH
Thioindoxyls	—	DP; ICI; CB
Thiolacetic acid (Thioglycollic acid)	92·12	BDH
DL-Thioctic acid (α-Lipoic acid)	—	LL; CC
Thionyl chloride	118·98	—
Thiophosgene	115	GN; RA
Thiosemicarbazide	91·14	BDH; LL
Thiosemicarbazone	236·29	GN
DL-α-Tocopherol acetate	472·73	R
o-Tolidine	212·3	BDH
Toluene-3,4-dithiol (Dithiol)	156·27	BDH
Transacetylase	—	SIG; LL
Trichloroacetic acid	163·4	—
Triketohydrindene hydrate	178·15	BDH; LL
1,5-bis(4-trimethylammoniumphenyl) pentan-3-one di-iodide	608	W
Triphenyl tetrazolium chloride	334	LL; MB; BDH
Triosephosphoric acid	—	B
Tris (Hydroxymethyl) aminomethane	121·14	BDH; EK; CC; LL
Triphosphopyridine nucleotide (TPN)	734·4	B; CC; SIG; LL
Triphosphopyridine nucleotide (reduced) (TPNH)	—	B; CC; SIG; LL
Trypsin	—	AR; SIG; LL
Trypsinogen	—	SIG; LL
Tryptamine	160	—
Tryptamine hydrochloride	196·69	LL; SIG; CC; BDH

* See List of Sources, p. 966.

Compound	M.W.	Source *
Tyramine hydrochloride	173·65	BDH; LL; CC
Uranyl nitrate ($6H_2O$)	502.18	BDH
Urease	—	LL; SIG
Vinyl alcohol (Polymerized)	—	BDH

* See List of Sources, p. 966.

ALPHABETICAL LIST OF SOURCES

Abbrev.	Full Description
AH .	Allen and Hanburys Ltd., Bethnal Green, London, E.2, England.
AL .	Aldrich Chemical Co. Inc., 3747, N. Booth Street, Milwaukee, 12, Wisconsin, U.S.A.
AR .	Armour Laboratories, Hampden Park, Eastbourne, England.
B. .	C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany.
BDH .	British Drug Houses Ltd., Poole, England.
BIOS .	Bios Laboratories, 17 W. 60th Street, New York, U.S.A.
BL .	Farbenfabriken Bayer, Leverkusen, Germany.
CB .	CIBA Ltd., Basel, Switzerland.
CC .	California Corporation for Biochemical Research, 3625, Medford Street, Los Angeles 63, California, U.S.A.
DJ .	Borden Co. Chemical Division, 5000 Langdon Street, Philadelphia 24, Pennsylvania, U.S.A.
DP .	E.I. Du Pont de Nemours & Co. Inc., Wilmington, Delaware, U.S.A.
EG .	Edward Gurr Ltd., 42, Upper Richmond Road W., London, S.W.14, England.
EK .	Eastman Kodak Co., Rochester 3, New York, U.S.A.
ER .	Evans, Norman & Rais Ltd., Stockport, Cheshire, England.
GG .	George Gurr Ltd., 136, New Kings Road, London, S.W.6, England.
GN .	British Biochemicals and Biologicals Ltd.* (Genatosan Division), Loughborough, Leicestershire, England.
HL .	Hartmann-Leddon Co. Inc., 5821, Market Street, Philadelphia 39, Pennsylvania, U.S.A.
HW .	Hopkin & Williams Ltd., Chadwell Heath, Essex, England.
ICI .	Imperial Chemical Industries Ltd., Dyestuffs Division, Blackley, Manchester, England.
LA .	Laporte Chemicals Ltd., Luton, Bedfordshire, England.
LL .	L. Light & Co. Ltd., Colnbrook, Buckinghamshire, England.
MB .	May & Baker Ltd., Dagenham, Essex, England.
NB .	Nutritional Biochemicals Corporation, 21010, Miles Avenue, Cleveland 28, Ohio, U.S.A.
PF .	Pfister Chemical Works, Ridgeway, New Jersey, U.S.A.
R. .	Roche Products Ltd., 15, Manchester Square, London, England.
RA .	Rapter Laboratories Inc., Chicago 54, Illinois, U.S.A.

* Now Whiffen and Sons Ltd.

Abbrev.	Full Description
SCH .	Schwarz Laboratories Inc., 230, Washington Street, Mount Vernon, New York, U.S.A.
SIG .	Sigma Chemical Co., 3500, Dekalb Street, St. Louis, Missouri, U.S.A.
T . .	Thorium Ltd., 10, Storey's Gate, London, S.W.1, England.
W .	Burroughs, Welleome & Co., Euston Road, London, N.W.1, England.
WC .	Watford Chemical Co. Ltd., 22, Copperfield Road, London, E.3, England.

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LONDON AND TONBRIDGE